Biochemical studies on the formation of bamboo lignin(Dissertation_全文)

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BIOCHEMICAL STUDIES ON THE FORMATION OF BAMBOO LIGNIN

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CONTENTS

CHAPTER I

INTRODUCTION

Lignin is widely distributed in nature as a cell wall constituent of terrestrial vascular plants. It is a macromolecular substance occurring in the largest amount next to cellulose. The fact that lignin is absent from aquatic plants but present in the terrestrial ones, particularly plentiful in forest trees, indicates that lignin might have played an important biological role in historical processes of plant evolution. Because it can be considered that the presence of lignified supporting tissues such as xylem has enabled the vascular plants to develop such large upright forms as found in forest trees. Furthermore, the fact that among woody plants, broad-leaved trees (angiosperm) differ from coniferous trees (gymnosperm) in the presence of syringyl units in lignin molecules, seems to have an intimate relationship with their biochemical evolution.

As to the question, "What is lignin ?", a great deal of investigations have been performed and its physico-chemical properties are mostly established at present. Gathering from the historical background of research works on lignin, there seem to have been two academic trends in this field. One is concerned with elucidation of the chemical structure of lignin, as represented by the school of Freudenberg. The other is shown by the works of Neish's group who have been trying to elucidate the biochemical process of lignin formation occurring in various higher plants.

The former contributed to demonstration of the old Klason's hypothesis that lignin is a dehydrogenation polymer (DHP) of coniferyl alcohol or its related alcohols, yielding free radicals on peroxidase-catalyzed dehydrogenation. The "free radical theory" is being established as an essential principle of lignin formation in higher plants.

The latter, a group of plant biochemists not only contributed to the elucidation of the biosynthetic pathways leading to lignin but also provided a biosynthetic idea which is helpful to understand the chemical structure of lignins. However, the biosynthetic pathways proposed at present are schematized on the basis of the results obtained mainly with tracer techniques using ^{14}C-labeled compounds. Therefore, more works with enzyme techniques (demonstration of the occurrence of the enzymes involved in biosynthesis of lignin) are needed in order to establish the proposed biosynthetic pathways that are still hypothetical.

The present investigations, from an aspect of plant biochemistry, have been performed in order to find a clue to the understanding of the mechanism of biochemical formation of lignins in higher plants. As a link in the chain of such research subjects, the following problems were taken up and investigated with growing bamboo as a representative plant material.

1. How the enzymes and the precursors are physiologically related with lignification of growing bamboo.

2. Whether or not ferulic acid is an obligate intermediate leading to sinapic acid.

3. What biochemical factors are involved in the formation of the different methoxyl groups in angiospermous and gymnosper-

mous lignins.

4. How bamboo lignin is characterized in chemical structure by the presence of p-coumaric acid esters.

Growing bamboo plants form an excellent plant material to study the changes in the occurrence of lignification intermediates and in the enzymatic system accompanying the successive stages of lignifcation in the maturing plant tissue.

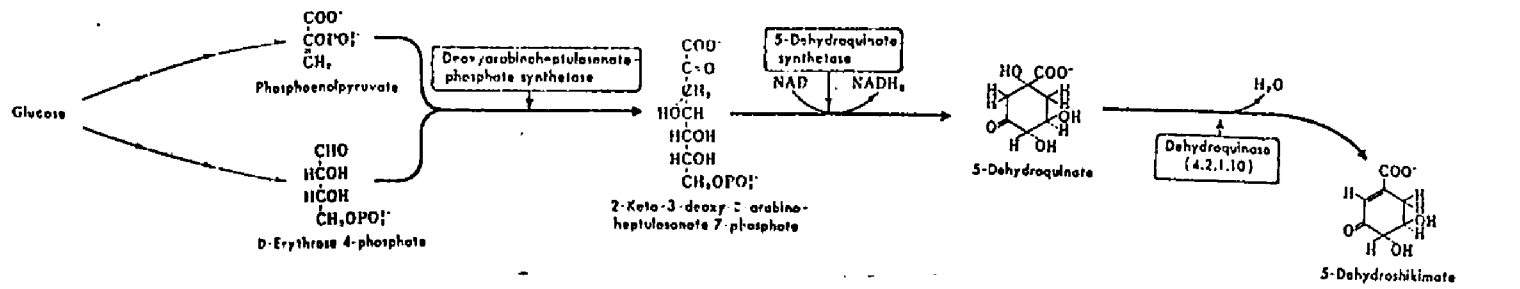
Glucose-6-phosphate:NADP-(G-6-P dehydrogenase; EC 1.1.1.49) and 6-phosphogluconate:NADP oxidoreductases (6-PG dehydrogenase; EC 1.1.1.44) involved in pentose phosphate pathway, shikimate: NADP oxidoreductase (dehydroshikimate reductase; EC 1.1.1.25) and dehydroquinate hydro-lyase (dehydroquinase; EC 4.2.1.10) in shikimate pathway, and S-adenosylmethionine:catechol O-methyltransferase (EC 2.1.1.6) in cinnamate pathway were extracted from the shoots of growing bamboo (Phyllostachys pubescens). These enzymes are found to play important roles in the biosynthesis of lignins as shown in "the metabolic pathways leading from glucose to lignin". CHAPTER II and III describe the characterization of these enzymes and the roles of them during lignification of bamboo shoots, respectively. The metabolism of lignin precursors such as shikimic acid, phenylalanine, tyrosine, p-coumaric acid (4-hydroxycinnamic acid) and ferulic acid (3-methoxy-4-hydroxycinnamic acid) are also investigated in relation to the lignin formation (CHAPTER III). The above-described enzyme works have been carried out concerning the first problem.

CHAPTER IV is concerned with the second problem.

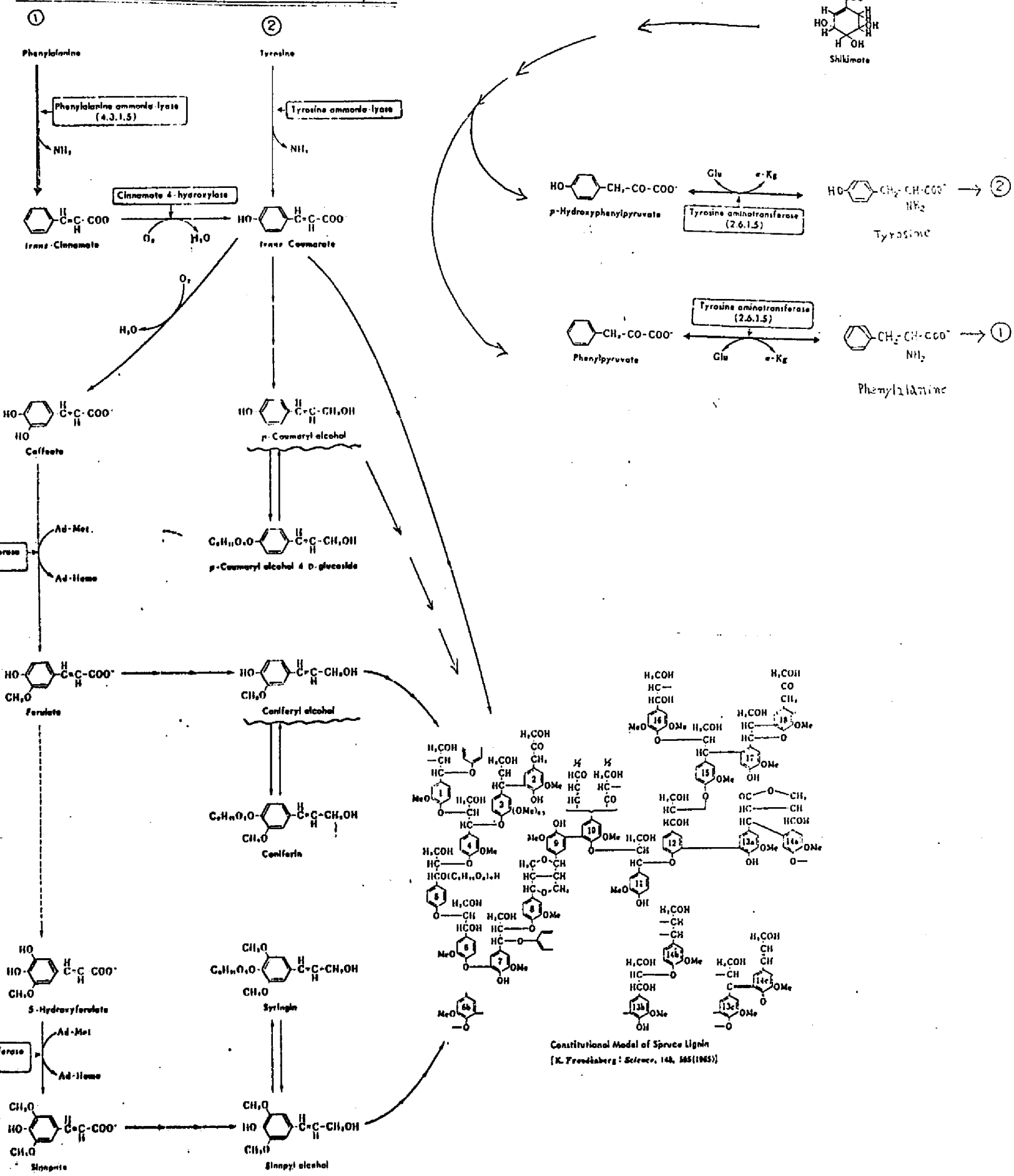
Ferulic acid has hitherto been considered as a natural intermediate of sinapic acid (4-hydroxy-3,5-dimethoxycinnamic acid)

METABOLIC PATHWAYS LEADING FROM GLUCOSE TO LIGNIN

Shikimate Pathway :



Phenylalanine-Cinnamate Pathway:



on the ground that both ferulic acid-2-^{14}C and 5-hydroxyferulic acid were incorporated into syringyl units of wheat lignin. However, no definite evidence to support such biosynthetic mechanism has yet been provided. Furthermore, it must be taken into account the occurrence of demethylation and demethoxylation of ferulic acid when this compound was administered to plants. Then, it is impossible to conclude only from the result obtained with ferulic acid labeled at the carbon of the side chain whether ferulic acid incorporated into syringyl units of lignin without removal of the methyl group or incorporated into them *via* caffeic acid (3,4-dihydroxycinnamic acid) after removal of the methyl group. Therefore, in order to find a clue to this problem, it is necessary to employ ferulic acid-O-$^{14}CH_3$, which was not tested in the earlier studies on lignin biosynthesis. This compound was successfully prepared from caffeic acid and S-adenosylmethionine-$^{14}CH_3$ by the mediation of bamboo *O*-methyltransferase. After feeding this labeled compound, the analytical data obtained by nitrobenzene oxidation and ethanolysis, supported the previous assumption that ferulic acid is a natural precursor of sinapic acid.

CHAPTER V is concerned with the third problem.

It is well known that as a rule angiosperm lignins consist of both guaiacyl and syringyl units whereas gymnosperm lignins hardly contain syringyl units but guaiacyl units. In addition, young xylem tissue of a growing plant contains very small amounts of syringyl units while older xylem tissue relatively large amounts of syringyl units. It is also interesting to learn that cultured callus tissues of angiosperms scarecely

give rise to syringaldehyde on nitrobenzene oxidation.

O-Methyltransferase may be regarded as one of the key enzymes that are involved in formation of syringyl lignin. Because bamboo and poplar O-methyltransferases were found to utilize both caffeic and 5-hydroxyferulic acid as substrates, yielding guaiacyl and syringyl components, respectively, whereas shoots of a ginkgo tree (gymnosperm) selectively methylated caffeic acid in the reaction mixture of both substrates. However, O-methyltransferase has not yet been isolated from gymnospermous plants. The author succeeded for the first time in extraction of this enzyme from seedlings of Pinus Thunbergii, the shoots of Ginkgo biloba, and the callus tissues of Salix caprea and mulberry (Morus bombycis). The substrate specificities of these O-methyltransferases are examined in relation to biochemical differences in the methoxyl patterns between angiosperm and gymnosperm lignins.

CHAPTER VI describes the ester linkages of p-coumaric acid in bamboo and grass lignins. Bamboo and grass contain 5-10% of p-coumaric acid esters in their lignins, which are found neither in angiospermous nor in coniferous plant lignin. Then, it is of interest to elucidate the structural pattern of the ester linkages of p-coumaric acid in graminae plant lignins with respect to their biochemical formation. However, their chemical structure is not yet established. On the basis of the analytical data obtained by methanolysis, thioglycolation and hydrogenolysis experiments with model compounds and natural lignins, it was proved plausible to believe that the majority of p-coumaric acid molecules are linked to the γ-position of the side chain of lignin molecules. Since the occurrence of p-coumaric acid esters is

regarded as a biological feature depending on plant species, the formation of these esters is considered to be biochemically controlled by the plants. Therefore, these esters may not be formed according to the mechanism of peroxidase-catalyzed reaction proposed by Kratzl and Okabe.

CHAPTER II

SEVERAL EXTRACTED ENZYMES INVOLVED IN BIOSYNTHESIS OF BAMBOO LIGNIN

PART 1. D-GLUCOSE-6-PHOSPHATE ; NADP AND 6-PHOSPHOGLUCONATE NADP OXIDOREDUCTASES

INTRODUCTION

It has been suggested that the pentose phosphate pathway plays an important role in the biosynthesis of aromatic compounds such as aromatic amino acids, flavonoids and lignins in higher plants(1,2), for these compounds are formed from shikimic acid which is synthesized from erythrose-4-phosphate and phosphoenolpyruvate provided through the pentose phosphate pathway and the glycolytic breakdown of glucose, respectively.

Several studies concerning resistant reaction of the plant tissues infected by certain pathogenic fungi have recently revealed that in the infected tissues the pentose phosphate pathway predominates, through which phenolic compounds, particularly lignin-like substances, are produced to protect the tissues against the penetration of the fungi (3,4).

There may be a similarity between the sugar metabolism leading to lignin formation in woody plants and that in the formation of phenolic compounds in the infected tissues. However, no direct evidence for individual enzymes of the pentose phosphate pathway in woody plants has yet been reported.

The present paper deals with the characterization of glucose-6-phosphate : NADP(G-6-P dehydrogenase; EC 1.1.1.49) and 6-phosphogluconate : NADP(6-PG dehydrogenase; EC 1.1.1.44) oxidoreductases extracted from bamboo shoots.

RESULTS AND DISCUSSION

Effect of pH on the enzyme activity :

The optimal pH of G-6-P and 6-PG dehydrogenases was found to be 8.0 and 8.5, respectively. The optimal pH values are approximately in agreement with those reported earlier with the enzymes from other higher plants (Figs. 1. and 2.) (5,6).

Effect of enzyme concentration on the rate of the reaction :

As shown in Fig. 3 the rate of reactions is proportional to the amount of enzyme solution added. Then, enzyme activity must be assayed within a linearly proportional range.

Specificity of enzyme :

As shown in Fig. 4, since no formation of $NADH_2$ occurred when NAD was added into the reaction mixture in place of NADP, both G-6-P and 6-PG dehydrogenases from bamboo shoot are NADP-specific. It was recently reported that G-6-P dehydrogenase from lettuce seedlings was active in the presence of NAD as well as of NADP, although crude enzyme preparation was used (7). When fructose-6-phosphate (F-6-P) was added in place of G-6-P and 6-PG, the formation of $NADPH_2$ was recognized by observing the increase in absorbance at 340 nm. The result probably indicates that F-6-P was utilized indirectly after conversion of F-6-P to G-6-P by G-6-P isomerase coexisting

with G-6-P dehydrogenase in the enzyme preparation used(Fig.5). Thus the activity pattern of G-6-P isomerase in growth will be studied by using a coupling system of G-6-P isomerase with G-6-P dehydrogenase.

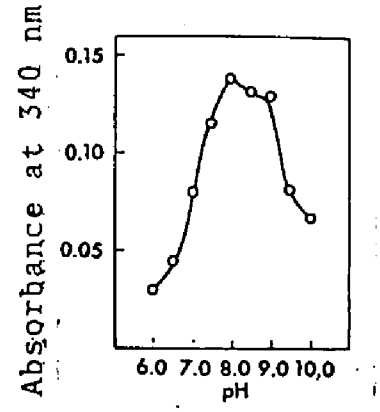


Fig. 1. EFFECT OF pH ON G-6-P DEHYDROGENASE.
0.05 M phosphate buffer(pH 6.0-7.5),
0.05 M Tris buffer(pH 7.5-9.0),
0.05 M glycine buffer(pH 9.5-10.5).
Enzyme concentration; 2.3 mg/ml.
Assay conditions were the same as described in the text except that buffers were altered as shown.

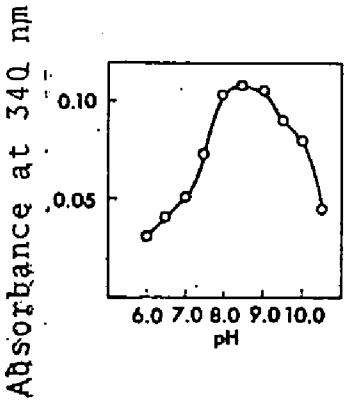


Fig. 2. EFFECT OF pH ON 6-PG DEHYDROGENASE.
0.05 M phosphate buffer(pH6.0-7.5),
0.05 M Tris buffer(pH 7.5-9.0),
0.05 M glycine buffer(pH 9.5-10.5).
Enzyme concentration; 2.3 mg protein/ml.

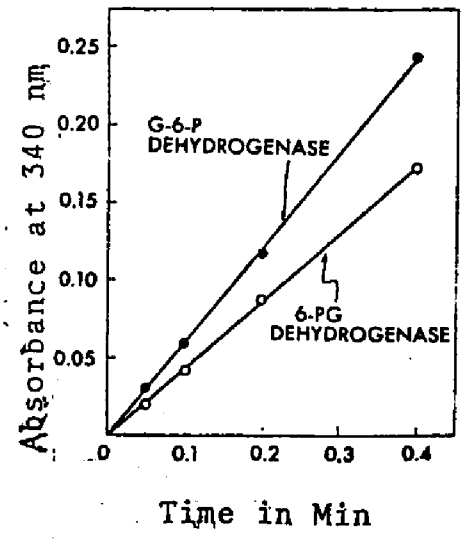


Fig. 3. EFFECT OF ENZYME CONCENTRATION ON THE RATE OF THE REACTION.
Enzyme concentration; 5.0 mg protein/ml.
Assay conditions were the same as described in the text.

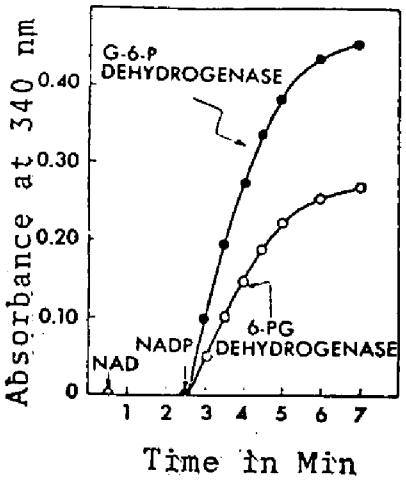


Fig. 4. EFFICIENCY OF NADP.

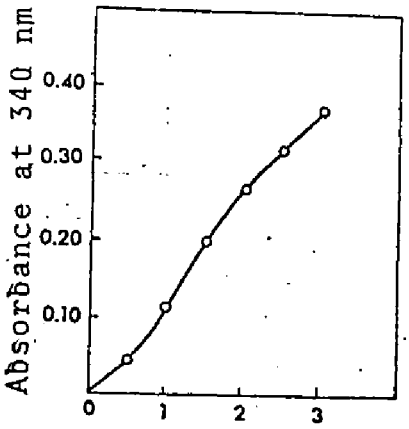


Fig. 5. INDIRECT UTILIZATION OF F-6-P.

MATERIALS AND METHODS

Plant materials :

Fresh bamboo shoots, Phyllostachys pubescens(Mohso) and Phyllostachys reticulata(Madake) were sampled from the Experimental Farm of Gifu University and used for the experiment.

Enzyme preparation :

After the sheath of immature bamboo of Mohso was removed, the stalk was cut into small pieces and blended in a Waring blender with its two times weight of 0.1 N sodium bicarbonate. The homogenate was strained through gauze and the filtrate was centrifuged at 4,000 rpm for 30 min at 0°. To the supernatant solution, solid ammonium sulfate was added to 0.4 saturation with stirring. The resulting precipitate was discarded after centrifugation. And again to the supernatant solution solid ammonium sulfate was added to 0.6 saturation. The precipitate was collected by centrifugation in the same way. The collected precipitate was dissolved in 20 ml of distilled water and dialyzed against water overnight at 0-4°. The dialyzate was used for assay after centrifugation at 10,000 rpm for 20 min.

With this enzyme preparation, the optimal pHs of G-6-P and 6-PG dehydrogenases were determined and the effect of enzyme concentration on the rate of the reaction and specificity of the enzymes were studied.

Assay of enzyme activity :

Since both G-6-P and 6-PG dehydrogenases require NADP as a coenzyme, the reaction was followed spectrophotometrically by measurement of increase in absorbance at 340 nm of $NADPH_2$ formed at room temperature using a Hitachi-Perkin Elmer UV-VIS spectrophotometer. Into a quartz cuvette of 1 cm light-path pipetted 0.20 ml of NADP ($2x10^{-3}M$), 1.0 ml of 0.05 M Tris buffer (pH 8.0), 0.20 ml of magnesium chloride ($10^{-1}M$) and a suitable amount of enzyme solution(0.20 ml was used for assay of G-6-P dehydrogenase and 0.3 ml for assay of 6-PG dehydrogenase). After addition of distilled water to make up 3.4 ml,0.20 ml of G-6-P ($10^{-2}M$) or 6-PG ($10^{-2}M$) was added as substrate and stirred quickly. Then, the increase in absorbance at 340 nm was measured at 30 second intervals for 1 min. The enzyme activity was estimated in the range of linear increase in the absorbance. When the presence of endogeneous substrate in the enzyme preparation had effect upon the assay, enzyme activity was determined by subtracting the increase in absorbance of control system (minus substrate) from the increase in absorbance of complete system. A unit of enzyme activity was defined as the amount of enzyme producing a change of absorbance of 1.0 per minute. The activities of enzyme preparations from 6 different parts of bamboo shoot were expressed on g fresh weight for comparison (see PART 5).

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PART 2. 5-DEHYDROQUINATE HYDRO-LYASE

INTRODUCTION

Since the finding of 5-dehydroquinate hydrolyase(EC 4.2.1.10) catalyzing the interconversion of 5-dehydroquinic acid and 5-dehydroshikimic acid from Aerobacter aerogenes (1), the occurrence of the enzyme in various tissues of higher plants has been shown by several workers (2-4). The enzyme is suggested to play an important role in the " shikimic acid pathway " which is a main reaction system in the biosynthesis of naturally occurring aromatic compounds. The present paper describes some characterization of 5-dehydroquinate hydro-lyase isolated from young bamboo shoot, and the distribution of the enzyme in other woody plants. The results are discussed in connection with biosynthesis of lignin.

RESULTS AND DISCUSSION

Identification of reaction product ;

In the assay system described, the reaction was allowed to proceed to completion and the absorption spectrum of the reaction mixture was measured by a Hitachi Perkin Elmer UV-VIS 135 spectrophotometer. A peak was observed at 234-235 nm corresponding to the absorption peak of 5-dehydroshikimic acid. The presence of 5-dehydroshikimic acid in the reaction mixture was also demonstrated paper-chromatographically by comparison with the synthetic acid using the solvent system of benzyl-

alcohol-ter-butanol-isopropanol-water (3:1:1:1) containing 2% of formic acid (descending) and a mixture of n-butanol-acetic acid-water (4:1:1) (ascending), respectively. The Rf values and the color of the reaction product sprayed with sodium periodate solution and the mixture of sodium nitroprusside and piperazine (5) are summarized in Table 1.

Table 1. IDENTIFICATION OF REACTION PRODUCT

Compound	Color*	Rf,I	Rf,II
5-Dehydroshikimic acid	Yellow brown	0.55	0.46
Reaction product	Yellow brown	0.54	0.45

*, According to the method of E. Haslam et al (5).

I, Developed with the solvent system of benzyl alcohol-ter-butanol-isopropanol-water (3:1:1:1) containing 2% of formic acid.

II, Developed with n-butanol-acetic acid-water (4:1:1).

Effect of enzyme concentration on the reaction velocity :

A typical time course of the reaction and the relation between the rate of reaction and the enzyme concentration are shown in Fig.1 and Fig.2, respectively. These results show that the reaction rate or the enzyme activity was determined within the range proportional to the incubation time or to the amount of the enzyme solution used.

Effect of pH on the enzyme activity :

In the assay procedure described, the variation of the

enzyme activity at different pHs was measured, by using various buffer solutions. As shown in Fig.3 the enzyme was found to be active over a broad range of pH without a peculiar peak between pHs 6.0 and 8.0. These results are consistent with those of 5-dehydroquinate hydro-lyase from a cauliflower (2), which also showed a broad activity range of pHs between 6.5 and 9.0 and the marked decrease in activity at pH 10.0.

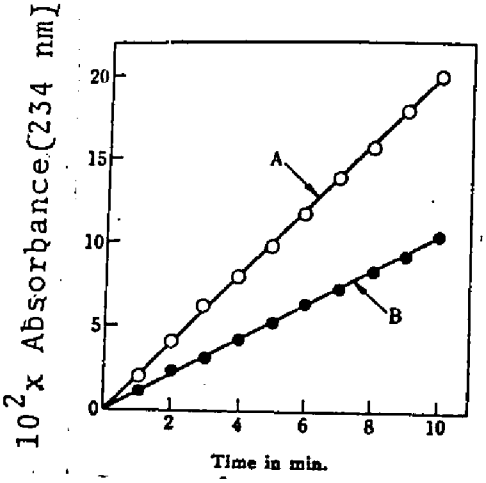

Fig. 1. TIME COURSE OF THE REACTION.
A; Enzyme concentration, 0.8 mg protein/0.1 ml.
B; Enzyme concentration, 1.2 mg protein/0.1 ml.
Enzyme preparations, A and B, were prepared separately at a different time.

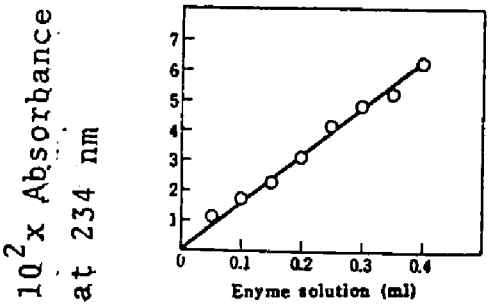

Fig. 2. EFFECT OF ENZYME AMOUNTS ON THE REACTION VELOCITY.

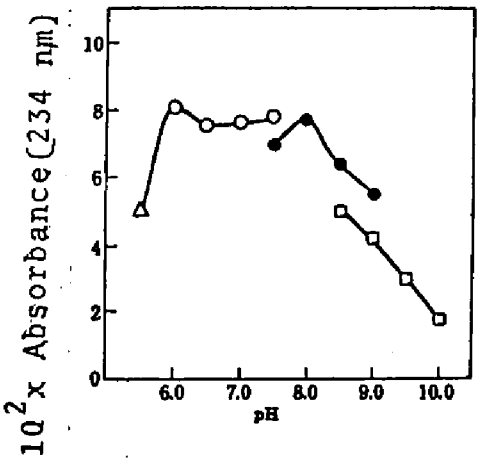

Fig. 3. EFFECT OF pH ON THE ENZYME ACTIVITY.
△, 0.05 M acetate buffer; -○-, 0.05M phosphate buffer; -●-, 0.05 M Tris buffer; -□- , 0.05 M glycine buffer.
Enzyme concentration; 0.5 mg protein/ 0.05 ml.
Assay conditions were the same as described in the text.

Michaelis constant determination :

Michaelis constant (Km value) for 5-dehydroquinate was determined by the method of Lineweaver and Burk as shown in Fig.4. The Km for dehydroquinic acid was found to be 1.3×10^{-5}M at pH 7.4, which was quite similar to the Km values of 4.1×10^{-5}M for bacterial enzyme (1) and 6.1×10^{-5}M for the cauliflower enzyme (2).

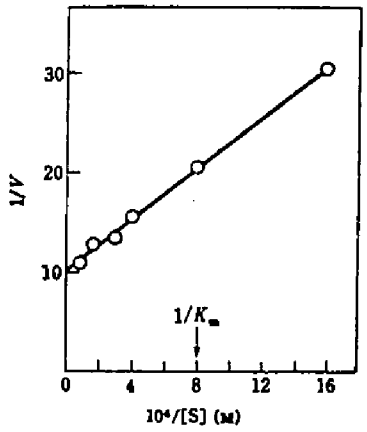

Fig.4. LINEWEAVER-BURK PLOTS FOR 5-DEHYDROQUINIC ACID.

Assay conditions were the same as described in the text except that 5-dehydroquinic acid concentration was altered as shown. Enzyme concentration, 0.8 mg protein/0.1 ml.

Distribution of 5-dehydroquinate hydro-lyase :

5-Dehydroquinate hydro-lyase was reported to be distributed widely in higher plants such as pea, spinach, cauliflower, cell suspension cultures of soy bean root, rose stem etc. In the present experiment the distribution of this enzyme in woody plants such as a young shoot of a tulip tree (Liliodendron tulipifera), seedlings of Pinus resinosa and Cryptomeria japonica, and asparagus (Asparagus officinalis) as well as bamboo shoots was surveyed.

No browning occurs in homogenates of monocotyledoneous plant tissues such as bamboo and asparagus, but homogenation of the tissues of a tulip tree and a poplar gave marked browning. Loomis and Battaile (6) recently reported that although on the homogenation of plant tissues in order to

extract enzymes most of them were inactivated by their binding with oxidized quinoid comounds derived from polyphenolic compounds, the inactivation was efficiently prevented by addition of Polyclar AT (insoluble polyvinylpyrolidone).

Table 2. DISTRIBUTION OF 5-DEHYDROQUINATE HYDRO-LYASE

Plant species	Activity (Unit/g fr. wt.)	(Unit/mg protein)
Bamboo shoot	0.35	9.8
Asparagus	0.06	0.11
Tulip tree*	1.90	23.6
Tulip tree**	0.16	1.5
Poplar*	negative	negative
Poplar**	negative	negative
Pinus resinosa	trace	0.03
Cryptomeria japonica	0.03	0.02

*, Tissues were homogenized with Polyclar AT (1/10 weight).
**, Tissues were homogenized without Polyclar AT.

The efficiency was recognized in the extraction of the enzyme from a tulip tree by comparison of the enzyme activities obtained with and without the addition of Polyclar AT. The results are listed in Table 2, indicating high enzyme activity in bamboo shoots and tulip trees.

MATERIALS AND METHODS

Samples and reagents :

Fresh immature bamboo shoots of Phyllostachys pubescens were sampled in the Experimental Farm of Gifu University and used for this experiment as an enzyme source.

5-Dehydroquinic acid and 5-dehydroshikimic acid were synthesized according to the method of Haslam, Howorth and Knowles (7).

Extraction of 5-dehydroquinate hydro-lyase :

All manipulations were carried out at 0-4° unless otherwise stated. The sheath of the bamboo shoot was removed and the shoot was cut into small pieces and blended in a Waring blender with an equal weight of 0.05 M potassium phosphate buffer (pH 7.4) containing 0.01 M sodium ascorbate. The homogenate was strained through gauze, the filtrate was centrifuged at 10,000 x g for 20 min. and the precipitate was discarded. To the supernatant solution, solid ammonium sulfate was added to 0.35 saturation. The precipitate was removed by centrifugation at the above-described centrifugal force and to the supernatant solution solid ammonium sulfate was added again to 0.50 saturation. The precipitate collected by centrifugation was dissolved in 0.05 M potassium phosphate buffer (pH 7.4) and passed through a Sephadex G-25 column (2.8x30 cm), previously equilibrated with the same buffer, to remove ammonium sulfate. The eluate was used for the assay of 5-dehydroquinate hydro-lyase.

Assay of enzyme activity :

Since 5-dehydroshikimic acid converted from 5-dehydroquinic acid by mediation of the hydrolyase has absorption peak at 234 nm, the reaction was followed spectrophotometrically by the measurement of increase in the absorbance at 234 nm. Into a quartz cuvette of 1 cm light-path were pipetted 3.0 ml of the phoshpate buffer (pH 7.4), a suitable amount of enzyme solution (0.05 or 0.1 ml usually used) and finally 0.1 ml of potssium dehydroquinate solution (0.5 μmole) and the reaction mixture was stirred quickly. Then, increase in the absorbance was measured at 1 minute intervals for 5 min. The enzyme activity was determined in the range of the linear increase in the absorbance. One unit of enzyme activity was defined as the amount of enzyme producing a change in the absorbance of 1.0 per minute at 234 nm and specific activity was defined as a unit per mg of protein. The concentration of protein in enzyme preparation was measured spectrophotometrically by the method of Warburg and Christian (9).

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PART 3. SHIKIMATE : NADP OXIDOREDUCTASE

INTRODUCTION

5-Dehydroshikimate reductase (shikimate:NADP oxidoreductase; EC 1.1.1.25) catalyzing the interconversion of dehydroshikimic acid and shikimic acid in the presence of NADP as a coenzyme was demonstrated in cell-free extracts of Escherichia coli by Yaniv and Gilvarg (1). In higher plants the presence of this enzyme was shown by Nandy and Ganguli (2), Balinsky and Davies (3) and Sanderson (4).

It has been suggested that shikimic acid plays an important role as a precursor of aromatic compounds, and a series of tracer experiments have shown the participation of the " shikimic acid pathway " in the biosynthesis of naturally occurring aromatic compounds such as aromatic amino acids, flavonoids and lignins in higher plants (5,6).

The present paper describes some characterization of 5-dehydroshikimate reductase isolated from immature bamboos.

RESULTS AND DISCUSSION

A typical example of the reaction catalyzed by dehydroshikimate reductase is shown in Fig. 1. The enzyme reaction was linear for 1 min, becoming slower thereafter and the reaction stopped after 5 min. As shown in Fig.2 the reaction was also followed by measurement of the increase in absorbance at 234 nm which is due to the absorption maximum of dehydroshikimic acid formed through dehydrogenation of shikimic acid.

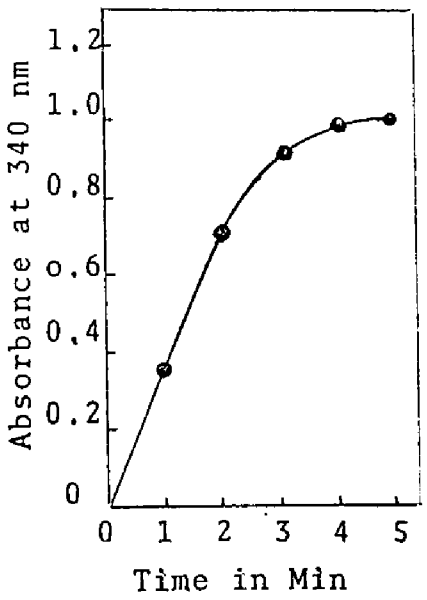

Fig. 1. TIME COURSE OF $NADPH_2$ FORMATION. Enzyme concentration; 6.7 mg protein/ml.

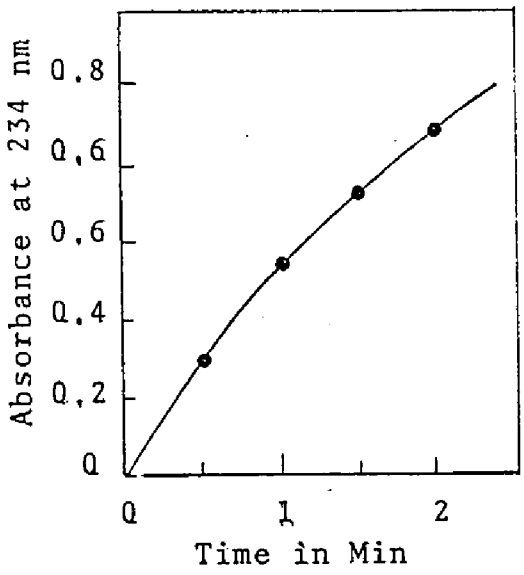

Fig. 2. TIME COURSE OF DHS FORMATION. Enzyme concentration; 6.2 mg protein/ml. DHS; dehydroshikimic acid.

Effect of pH :

The effect of pH on the rate of the enzyme reaction was examined by using various buffer solutions with different pHs. As shown in Fig. 3 the optimal pH of 5-dehydroshikimate reductase of bamboo shoot was unexpectedly quite alkaline (pH, 11.0) as compared with those of pea seedlings (pH, 10.0), mung bean seedlings (pH, 8.0) and tea plant (pH, 10.1). The activity sharply decreased in the more alkaline range over pH 11.0.

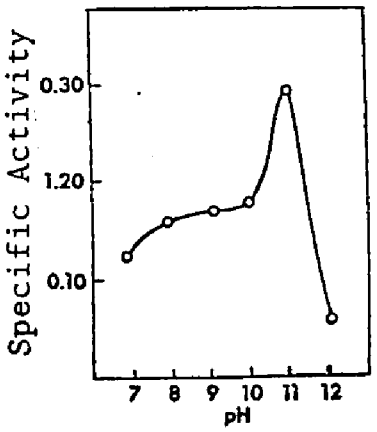

Fig. 3. EFFECT OF pH ON ENZYME ACTIVITY.
0.05 M phosphate buffer (pH 6.0-7.5)
0.05 M Tris buffer (pH 7.5-9.0)
0.05 M glycine buffer (pH 9.5-11.0)
0.05 M $NaOH$-Na_2HPO_4 buffer (pH 12.0)
Enzyme concentration; 3.7 mg protein/ml. Assay conditions were the same as described in the text except that buffers were altered as shown.

Specificity of enzyme :

The bamboo 5-dehydroshikimate reductase was found to be NADP specific because NADP could not be replaced by NAD, as shown in Fig. 4. Quinic acid was not utilized by this enzyme as shown in Fig. 5. These results are quite in accordance with the previously reported ones (1-4).

As can be seen in Figs. 4 and 5, the enzyme reaction was dependent on the concentration of NADP and shikimic acid. When the enzyme reaction was equilibrated and the increase of absorbance stopped, further addition of NADP or shikimic acid into the reaction mixture brought about the increase in the absorbance at 340 nm.

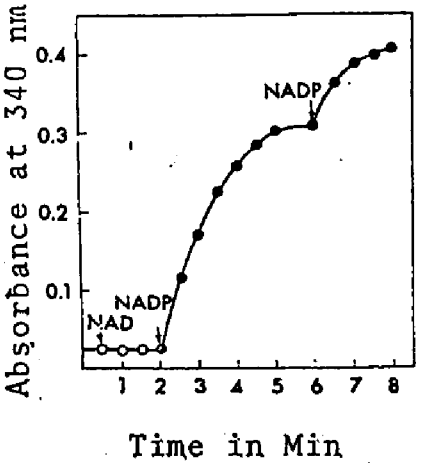

Fig. 4. SPECIFICITY OF ENZYME AND DEPENDENCE OF THE REACTION ON NADP CONCENTRATION.
Enzyme concentration; 13.2 mg protein/ml. NAD or NADP was added at the points shown.

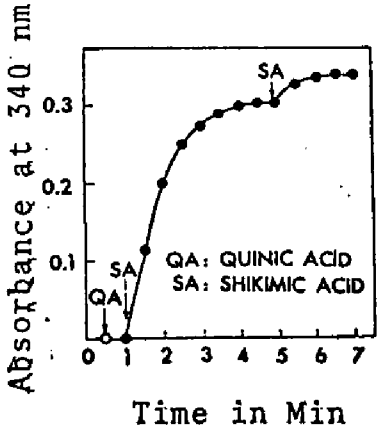

Fig. 5. SPECIFICITY OF ENZYME AND DEPENDENCE OF THE REACTION ON SHIKIMIC ACID CONCENTRATION.
Enzyme concentration; 9.8 mg/ml. Quinic acid and shikimic acid were added at the points shown.

Reversibility of the reaction :

The assay system described in the text was used. The reaction was allowed to proceed for 5 min, when the velocity had considerably slowed down. Dehydroshikimic acid (0.5 μmole) was then added, and the absorbance at 340 nm was found to decrease, owing to reoxidation of $NADPH_2$ by the acid added (Fig. 6). The addition of shikimic acid caused the absorbance to increase again.

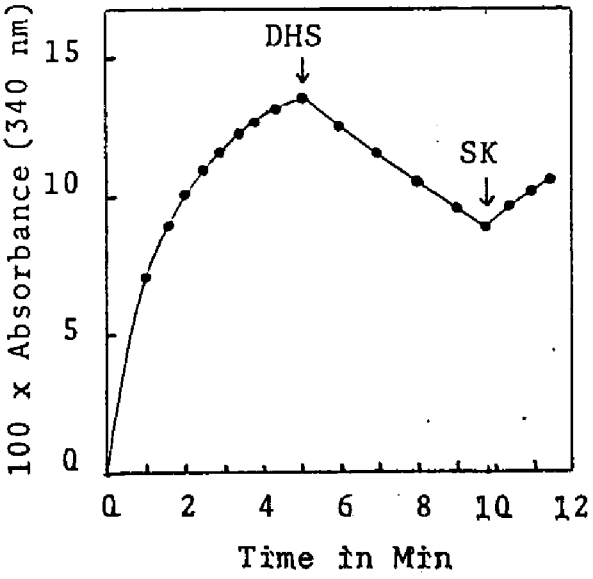

Fig. 6. DEMONSTRATION OF THE REVERSIBILITY OF THE REACTION. Assay conditions were as given in the text. DHS (dehydroshikimic acid) and SK (shikimic acid) were added at the points shown.

Michaelis constant :

Michaelis constants (Km) for the substrates were calculated by the method of Lineweaver and Burk, using a partially purified enzyme. The Km's for shikimic acid and NADP were found to be 2.0×10^{-4}M and 1.4×10^{-4}M, respectively at pH 8.0 (Figs. 7 and 8).

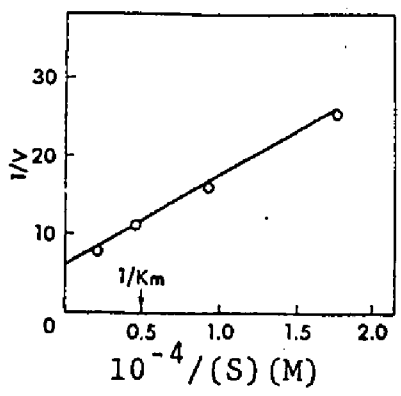

Fig. 7 LINEWEAVER-BURK PLOTS FOR SHIKIMIC ACID. A purified enzyme preparation was used. Assay conditions were the same as described in the text except that shikimic acid concentration was altered as shown. Enzyme concentration; 11.3 mg protein/ml.

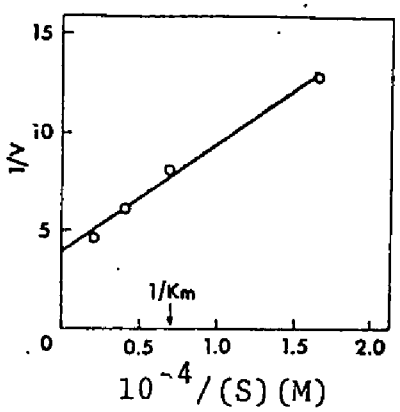

Fig. 8. LINEWEAVER-BURK PLOTS FOR NADP. A purified enzyme preparation was used. Assay conditions were the same as described in the text except that NADP concentration was altered as shown. Enzyme concentration; 11.3 mg protein/ml.

Effect of various inhibitors on the enzyme activity :

Effects of ethylenediamine tetraacetic acid (EDTA), sodium fluoride, sodium arsenite and p-chloromercuribenzoic acid on

Table 1. EFFECT OF VARIOUS COMPOUNDS ON ENZYME ACTIVITY

Compounds	Concentration (mM)	Activity (% of original)
EDTA*	1.0	95
NaF	1.0	100
Sodium arsenite	1.0	100
PCMB**	1.0	44

*, Ethylenediamine tetraacetic acid.

**, p-Chloromercuribenzoic acid.

enzyme activity were examined. Little change in enzyme activity was observed on addition of EDTA, indicating no metal requirement. On the other hand, p-chloromercuribezoic acid inhibited more than half of the activity, which might mean that the enzyme has sulfhydryl group in an active center (Table 1). This inhibition obtained is in accordance with the results previously reported.

Distribution of 5-dehydroshikimate reductase in woody plants :

5-Dehydroshikimate reductase was extracted from stem of udo (Aralia cordata), asparagus shoots (Asparagus officinalis) and a young branch of tulip tree (Liriodendron tulipifera) . The pattern of the reaction catalyzed by the 5-dehydroshikimate reductase from different species was recognized to be similar to that shown in Fig. 1. Tulip trees are also considered to be good materials for the enzymatic studies on lignification of trees. This enzyme has been isolated from the tea plant (Camellia sinensis) and characterized by Sanderson (4).

MATERIALS AND METHODS

Plant materials :

Bamboo shoots, udo shoots and asparagus shoots were sampled in the Experimental Farm of Gifu University. Branches of tulip trees were taken in the campus of the university.

Extraction of 5-Dehydroshikimate rductase :

All manipulations were carried out at 0-4° unless otherwise stated. After removal of sheath of bamboo shoots, the shoots

were cut into small pieces and homogenized with an equal weight of 0.01 M phosphate buffer (pH 7.0). The homogenate was strained through gauze, the filtrate was centrifuged at 4,000 rpm for 30 min, and the precipitate was discarded. The supernatant solution was adjusted to pH 5.0 with M acetic acid and the resulting precipitate was removed by centrifugation. To the supernatant solution, cold acetone (-15°) was added carefully with stirring to 50% concentration. The precipitate was collected by centrifugation and dissolved in 0.05 M Tris buffer (pH 8.0). After centrifugation at 10,000 rpm for 30 min, the supernatant solution was used for assay of the enzyme.

Enzyme assay :

The reaction catalyzed by the enzyme was followed by measurement of absorbance at 340 nm of $NADPH_2$ formed at room temperature using a Hitachi Perkin-Elmer spectrophotometer. Into a quartz cuvette of 1 cm light-path were pipetted 0.25 ml of NADP (2×10^{-3}M), 1.0 ml of 0.05 M Tris buffer (pH 8.0) and a suitable amount of enzyme solution. After addition of distilled water to make up 3.0 ml, 0.5 ml of shikimic acid (6×10^{-3}M) was added as a substrate and the mixture was stirred quickly. Then, the increase in absorbance at 340 nm was measured immediately. The enzyme activity was estimated in the range of linear increase in the absorbance. A unit of enzyme activity was defined as the amount of the enzyme producing a change of absorbance of 1.0 per mg protein.

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PART 4. S-ADENOSYLMETHIONINE : CATECHOL O-METHYLTRANSFERASE

INTRODUCTION

In plant kingdom are many kinds of methoxyl aromatic compounds such as lignins, lignans, flavonoids and alkaloids. Incorporation of methyl group of methionine into methoxyl groups of lignins was first demonstrated by Byerrum *et al* (1), using barley plants administered with methionine-methyl-^{14}C and they pointed out the role of methionine in enzymatic methoxylation of lignins in higher plants.

Recently, Finkle and Nelson (2) reported the occurrence of S-adenosylmethionine:catechol O-methyltransferase (EC. 2.1.1.6) extracted from cambial tissues of an apple tree, and the characterization of O-methyltransferase extracted from pampas grass (3).

As to the investigation of lignin biosynthesis it was suggested that cinnamic acid derivatives with methoxyl groups such as ferulic, 5-hydroxyferulic and sinapic acids were potent lignin precursors in tracer experiments with radioisotopes (4). However, the role of S-adenosylmethionine:catechol O-methyltransferase in lignification of woody plants and its enzymic properties have not yet been elucidated. Higuchi *et al*. indicated the possible participation of O-methyltransferase in lignin formation since they recognized methoxyl content increased with proceeding of lignification of bamboo shoots (5).

The present paper describes the extraction of O-methyl-

transferase from bamboo shoots and its characterization. "Methionine activating enzyme", ATP:L-methionine S-adenosyltransferase (EC. 2.5.1.6) from the shoots is also described briefly.

RESULTS AND DISCUSSION

Identification of reaction product :

In the assay system described the reaction products were identified by paper chromatography using a solvent system of upper layer of toluene-acetic acid-water (4:1:5) and lower layer of chloroform-acetic acid-water (2:1:1), respectively. The reaction product was identified as ferulic acid by comparison of its Rf value with that of an authentic compound and the ultraviolet absorption spectrum of the reaction product was identical to that of authentic ferulic acid as shown in Fig. 1.

Time course of ferulic acid formation :

As shown in Fig. 2, ferulic acid was formed almost linearly within 30 min and no marked increase in yield of the acid was recognized by the further incubation. The amount of ferulic acid formed enzymatically in the complete reaction system and in the control systems is shown in Table 1. In the control systems without addition of each of caffeic acid, S-adenosylmethionine and enzyme, no formation of ferulic acid was observed. In the system without $MgCl_2$, however, a considerable amount of ferulic acid was produced although the yield was lower than that in the complete system. This indicates that the presence of magnesium ion in the reaction mixture is necessary for maxi-

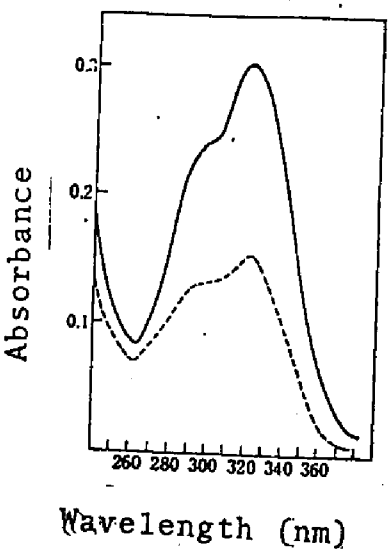

Fig. 1. UV-SPECTRA OF FERULIC ACID AND REACTION PRODUCT IN EtOH.
—— ; ferulic acid,
---- ; reaction product.

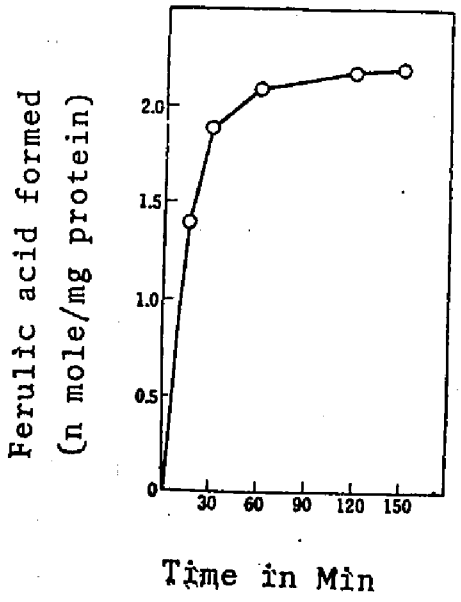

Fig. 2. TIME COURSE OF FERULIC ACID FORMATION.
Enzyme; 30 mg protein was used for standard assay as described in the text.

TABLE I. ENZYMIC FORMATION OF FERULIC ACID

System	Ferulic acid formed ($\times 10^{-3}$ μmole/mg protein)	Ratio
*Complete	4.1	100
Minus caffeic acid	0	0
Minus S-AMe	0	0
Minus $MgCl_2$	3.2	78
Minus enzyme	0	0

* Assay conditions were described in the text, and 30.8 mg of enzyme protein was used for assay.
S-AMe; S-Adenosylmethionine.

mum formation of ferulic acid.

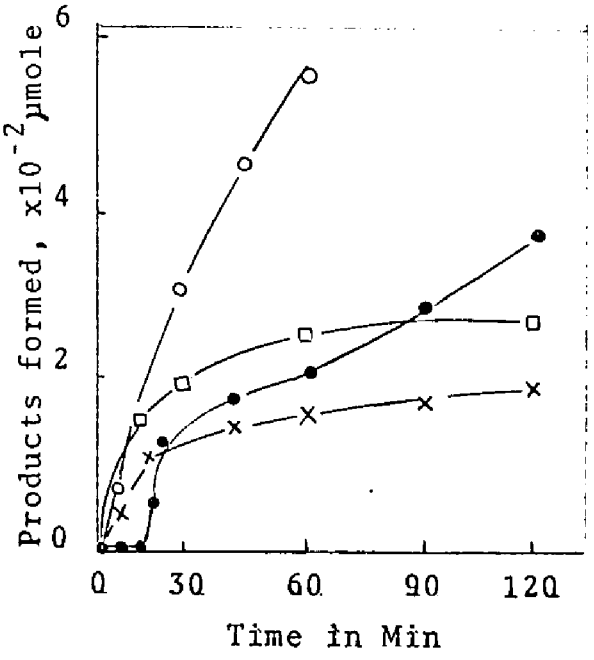

Fig. 3. TIME COURSE OF FORMATION OF METHYLATED HYDROXYCINNAMIC ACIDS.
□; ferulic acid,
○; sinapic acid from 5-hydroxyferulic acid,
×; 5-hydroxyferulic acid from 3,4,5-trihydroxycinnamic acid,
◉; sinapic acid from 3,4.5-trihydroxycinnamic acid.

Figure 3 shows the time course for formation of O-methylated hydroxycinnamic acids by O-methyltransferase from bamboo shoot. The rate of formation of sinapic acid (SA) from 5-hydroxyferulic acid (HF) was found to be much greater than that for ferulic acid (FA) from caffeic acid (CA). 3,4,5-Trihydroxycinnamic acid (TC) was also efficient as a substrate and gave both HF and SA; for the first 10 min after the start of the enzymic reaction only HF was formed from TC. However, when SA formation begins, the rate is higher than that of HF. Thus, the lag phase observed in the formation of SA suggests that the two meta-hydroxyl groups of TC are not methylated at the same time, but that HF is an intermediate.

Effect of pH on enzyme activity:

In the assay procedure described, the variation of enzyme activity at different pH values was measured using various buffer solutions. As shown in Fig. 4, the optimum pH was found to be 8.0 which was quite similar to the results obtained with the enzyme from an apple tree (optimal pH 7.0-8.0) (2) and the enzyme from rat liver (optimal pH 7.5-8.2) (6).

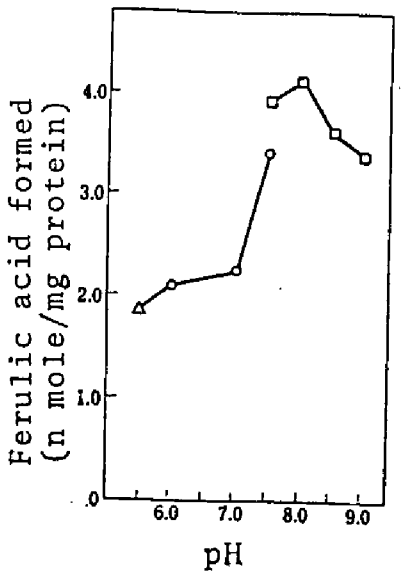

Fig. 4. EFFECT OF pH ON ENZYME ACTIVITY.
△; 1 M acetate buffer
○; 1 M phosphate buffer
□; 1 M Tris buffer
Assay conditions were the same as described in the text except that buffers were altered as shown. Enzyme; 30.8 mg protein was used for the assay.

Michaelis constant ;

Michelis constants (Km) for caffeic acid obtained by the Hofstee and by Lineweaver-Burk methods were 3.5×10^{-5} M and

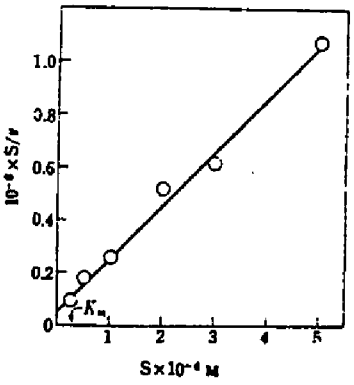

Fig. 5. Km VALUE FOR CAFFEIC ACID BY HOFSTEE METHOD.
Assay conditions were the same as described in the text except that substrate concentration was altered as shown. 34.2 mg enzyme protein from P. reticulata was used.

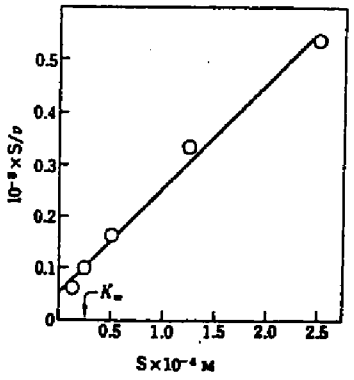

Fig. 6. Km VALUE FOR S-ADENOSYLMETHIONINE BY HOFSTEE METHOD.
Assay conditions were the same as described in the text except that substrate concentration was altered as shown. Enzyme concentration; 34.2 mg protein was used.

$4x10^{-5}$M, respectively at pH 8.0 in the case of the enzyme preparation from Phyllostachys pubescens (Mohso). However, in the course of this experiment Mohso bamboos were out of season, and thus, Madake (Phyllostachys reticulata) was used as an enzyme source. In this case Km values for caffeic acid and S-adenosylmethionine were found to be $3x10^{-5}$ M and $2.5x10^{-5}$ M, respectively at pH 8.0. The results are shown in Figs. 5 and 6.

Inhibition experiments:

Effects of p-chloromercuribenzoic acid (PCMB), EDTA and mono-iodoacetic acid on enzyme activity were examined as given in Table 2, PCMB inhibited the enzyme considerably, and EDTA

Table 2. INHIBITION EXPERIMENTS

System	Original activity (%)
Complete without inhibitors*	100
Plus PCMB (3 mM)	66
Plus PCMB (0.3 mM)	90
Plus EDTA (10 mM)	80
Plus iodoacetic acid (3 mM)	106
Plus iodoacetic acid (0.3mM)	100

*, Assay conditions were described in the text.

showed slight inhibition. However, the enzyme was not inhibited by iodoacetic acid which is known as one of the potent inhibitors for sulfhydryl group of enzymes. This is probably because the enzyme preparation used for assay contained a large amount

of other proteins which prevented the enzyme from inhibition by iodoacetic acid. Anyway, the fact that O-methyltransferase was inhibited by PCMB is consistent with the results reported previously, showing that the bamboo O-methyltransferase should be one of the enzymes with sulfhydryl group as an active center.

Stabilization of the enzyme :

The bamboo O-methyltransferase was found to be quite unstable because most enzyme activity was lost during some fractionation procedures. More than 70% of the enzyme was inactivated on $(NH_4)_2SO_4$ fractionation. However, this inactivation was prevented by addition of EDTA and SH-reagents before the fractionation. Fig. 7 shows the effectiveness of SH-reagents such as cysteine, mercaptoethanol and Clerand's reagent (dithiothreitol)

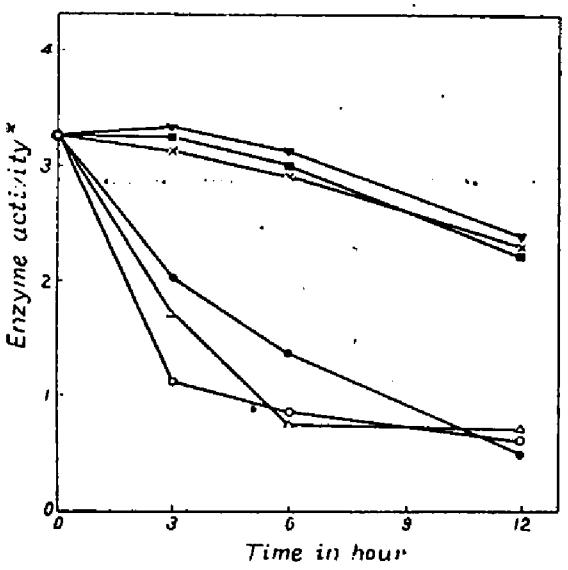

Fig. 7. STABILITY OF THE ENZYME AT 15°.

▲, cysteine added,
■, Clerand's reagent added,
×, mercaptoethanol,
●, ascorbate added,
○, caffeic acid added,
△, glycine added.
*, The activity is expressed as amounts of ferulic acid formed (10 n moles).

for the stabilization of the enzyme. When the enzyme was stored in the solution containing mercaptoethanol (2 mM) at 4° , the activity was retained for at least 2 days.

The occurrence of ATP:L-methionine adenosyl transferase (methionine activating enzyme) :

The "methionine activating enzyme", ATP:L-methionine adenosyl transferase (EC 2.5.1.6) was for the first time extracted from barley plants by Mudd (7), indicating that S-adenosylmethionine is an important intermediate on the methylation in higher plants. As already described above, S-adenosylmethionine was found to serve as a methyl group donor for hydroxycinnamic acids such as CA, HF, and TC. Therefore, the methionine activating enzyme should really be present in the bamboo shoots. In fact, this enzyme was cell-free extracted from acetone powder of the shoots of both Phyllostachys pubescens and Phyllostachys reticulata. However, this enzyme could not be extracted in an active form by buffer extraction.

Fig. 8 shows that radioactive S-adenosylmethionine was formed from ATP and methionine-$^{14}CH_3$ by mediation of the activating enzyme. Furthermore, Fig. 9 shows that radioactive ferulic acid was enzymatically formed from CA in the presence of ATP and methionine-$^{14}CH_3$.

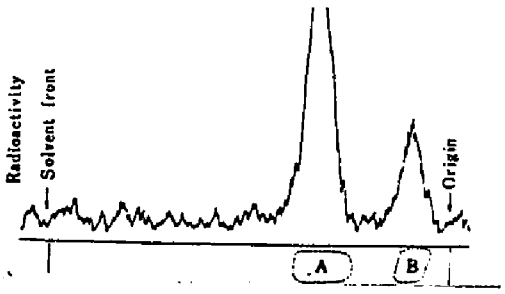

Fig. 8. RADIOCHROMATOGRAM OF S-ADENOSYLMETHIONINE ENZYMATICALLY FORMED.
Spots A and B correspond to methionine and S-adenosylmethionine, respectively, detected by ninhydrin color reaction. A solvent system for paper chromatography; n-BuOH-AcOH-H_2O (5;1:4, organic layer).

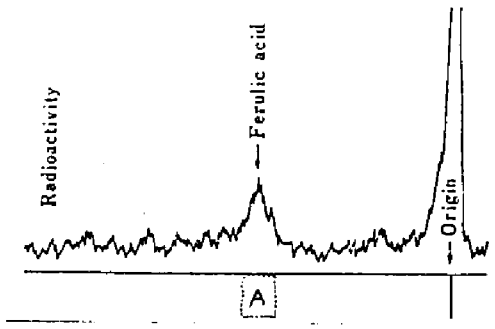

Fig. 9. RADIOCHROMATOGRAM OF FERULIC ACID FORMED FROM CAFFEIC ACID IN THE PRESENCE OF ATP AND METHIONINE-$^{14}CH_3$.

A spot A was found to be identical with authentic FA by color reaction of p-nitroaniline reagent after paper chromatography with a solvent of toluene-AcOH-H_2O (4:1:5, upper layer).

These rsults show that methionine activating enzyme also occurs in growing bamboo shoots, indicating that the enzyme plays an important role in formation of methoxyl groups of lignin precursors, and cosequently, in lignification of plants. In addition, the fact that the methyl group of methionine was transferred to meta-hydroxyl group of CA is in good accordance with the early report on the incorporation of the methyl group of methionine into the methoxyl groups of lignins (1).

Substrate specificity of bamboo O-methyltransferase:

In relation to mechanism of formation of guaiacyl and syringyl groups of lignins, it is quite important to investigate substrate specificity of the bamboo O-methyltransferase. Because this is possibly correlated with differences of methoxyl group patterns between angiospermous and gymnospermous lignins. Such differences of the methoxyl groups involving O-methyltransferases from different species of plants are discussed in more detail in CAHAPTER VI.

Rf values and color reactions of substrates tested and the expected products were listed in Table 3, which was used as criteria for identification of the expected methylated products. As the result Table 4 shows how the various phenolic compounds

Table 3. Rf VALUES AND COLOR REACTIONS OF AUTHENTIC COMPOUNDS

Compounds	R_f values			Colours		
	(1)*	(2)*	(3)*	In u.v.	(A)†	(B)†
p-Coumaric acid	0·04	0·53	0·14	dark violet	grey	red-brown
p-Methoxycinnamic acid	0·83	0·71	—	dark grey		
Caffeic acid	0	0·28	0·50	blue	yellow-brown	brown
Ferulic acid	0·35	0·39	0·97	bright blue	blue	red-violet
Isoferulic acid	0·15	0·27	—	blue-violet	violet	red-brown
3,4-Dimethoxycinnamic acid	0·72	0·43	—	faint blue		
3,4,5-Trihydroxycinnamic acid	0	0·17	0	blue	yellow-brown	yellow-brown
5-Hydroxyferulic acid	0	0·21	0·21	light blue	grey	dark brown
Sinapic acid	0·25	0·27	0·98	green-blue	grey-brown	pink
	(1)*	(4)*	(5)*	In u.v.	(B)†	(C)†
p-Hydroxybenzaldehyde	0·27	0·35	—			yellow-brown
Anisaldehyde	0·96	0·89	—	dark		bright yellow
Protocatechualdehyde	0	0·01	0·28			yellow-brown
Vanillin	0·84	0·78	0·84			yellow-brown
Isovanillin	0·59	0·67	0·78	light blue	yellow	yellow-brown
Veratraldehyde	0·80	0·92	0·92	blue	bright yellow	yellow-brown
5-Hydroxyvanillin	0·08	—	0	dark		brown-orange
Gallaldehyde	0	—	0	dark		brown-orange
Syringaldehyde	0·80	—	0·10	dark		brown-orange
	(4)*		(5)*	In u.v.	(B)†	(D)†
p-Hydroxybenzoic acid	0·01		0·28			bright yellow
Anisic acid	0·74		0·84			bright yellow
Protocatechuic acid	0		0·02		red-blue	grey-green
Vanillic acid	0·08		0·42		yellow	yellow-brown
Veratric acid	0·67		0·78			yellow
Gallic acid	0		—		bright yellow	grey-blue
Syringic acid	0·88		—		blue-grey	grey-brown
Pinosylvin	0·27		—	light blue	orange-yellow	
Pinosylvin monomethyl ether	0·90		—	light blue	yellow	

* Solvents used for paper chromatography: (1) toluene–AcOH–H_2O (4:1:5, organic layer); (2) 2% AcOH; (3) $CHCl_3$–AcOH–H_2O (2:1:1, organic layer); (4) benzene–HCOOH–H_2O (500:1:49, organic layer); (5) benzene–MeCOEt–HCOOH–H_2O (450:50:1:49, organic layer).

† Colour reagents: (A) diazotized *p*-nitroaniline; (B) diazotized sulphanilic acid; (C) 2,4-dinitrophenylhydrazine in 2 N HCl solution; (D) 2% $FeCl_3$.

Table 4. SPECIFIC METHYLATION BY BAMBOO O-METHYLTRANSFERASE.

Substrate	Expected product	Result
Caffeic acid	Ferulic acid	+
5-Hydroxyferulic acid	Sinapic acid	+
3,4,5-Trihydroxycinnamic acid	5-Hydroxyferulic acid	+
3,4,5-Trihydroxycinnamic acid	Sinapic acid	+
Chlorogenic acid*	Feruloylquinic acid	+
p-Coumaric acid	*p*-Methoxycinnamic acid	–
Caffeic acid	Isoferulic acid	–
Isoferulic acid	3,4-Dimethoxycinnamic acid	–
p-Hydroxybenzoic acid	Anisic acid	–
Protocatechuic acid	Vanillic acid	–
Protocatechuic acid	Veratric acid	–
Gallic acid	Syringic acid	–
p-Hydroxybenzaldehyde	Anisaldehyde	–
Protocatechu-aldehyde	Vanillin	–
Protocatechu-aldehyde	Isovanillin	–
Isovanillin	Veratraldehyde	–
5-Hydroxyvanillin	Syringaldehyde	–
Gallaldehyde	5-Hydroxyvanillin	–
Gallaldehyde	Syringaldehyde	–
Pinosylvin	Pinosylvin monomethylether	–

* The reaction mixture containing chlorogenic acid (1 μmole) as a substrate was incubated for 30 min at 30° as described in the text. After the addition of 0·5 ml of 10% HCl, the mixture was extracted with 20 ml EtOAc. The EtOAc fraction was divided into two equal portions. One was submitted to paper chromatography in toluene–AcOH–H_2O (4:1:5, upper layer) in order to determine free ferulic acid originally contained in the reaction mixture; however, none was present. The other portion was submitted to HCl-hydrolysis and ferulic acid liberated from any enzymatically formed feruloylquinic acid was determined as described in the text. The amount of ferulic acid after hydrolysis was found to be 2·2 μg.

were methylated. As already shown in Fig. 3, CA, HF and TC were efficiently methylated, yielding the respective products. Since TC was found to be a considerably potent substrate, it may also act as a precursor of SA in the biosynthesis of lignins on the assumption that CA is hydroxylated at 5-position to TC during lignin formation. However, this compound has not yet been detected from plant kingdom but as a moiety of a B-ring of flavonoids and their derivatives (8). Chlorogenic acid (caffeoylquinic acid) was also methylated, giving feruloylquinic acid. On the other hand, p-hydroxycinnamic acid and iso-FA (3-hydroxy-4-methoxycinnamic acid) were not methylated. Similarly, neither of iso-FA

and 3,4-dimethoxycinnamic acid was formed from CA. Other phenolics such as benzoic acids and benzaldehydes were not utilized for the enzymatic methylation. These results support the early investigaions on efficiencies of lignin precursors with tracer experiments (4, 9-11). Although Hess (12) reported that protocatechualdehyde, protocatechuic and gallic acids and esculetin were methylated by cell-free extracts from petunia and Finkle et al. (3) observed a similar methylation with a pampas grass O-methyltransferase, the bamboo enzyme showed a narrow specificity only for 3,4-dihydroxycinnamic acid derivatives. However, the fact that the bamboo enzyme has meta-specificity to CA, HF and TC is in good accordance with the previous reports (3, 12). On the other hand, O-methyltransferase from animal liver was shown to methylate at both para- and meta-positions. And yet it must be considered that para-specific O-methyltransferases are really present in plants, as found in the enzymatic para-methylations of p-hydroxycinnamic acid in Foeniculum vulgare (13) and of norbelladine, a precursor of amaryllidaceae alkaloids (14). Therefore, meta- or para-specific methylation seems to depend on plant species. It is not clear what factors are involved in such a selective methylation. One of the factors may be a rquirement for a magnesium ion because para-specific enzyme does not require this cation (14) and a magnesium ion could affect enzyme-substrate orientation by cross-linking between p-hydroxyl group of catechols and S-adenosylmethionine, as proposed by Senoh et al.(15). Consequently, the meta-hydroxyl group is substituted by the methyl group of S-adenosylmethionine yielding meta-methylated products. Variations in the pH values of the reaction medium

can also influence the ratio of para and meta- methylated products in some compounds (15).

At any rate, in conclusion, the meta-specificity of the bamboo O-methyltransferase is quite compatible with the fact that lignin molecules consist of meta-methoxylated components such as guaiacyl and syringyl nuclei.

Purification studies:

Although CA and HF including TC were demonstrated to be potent methyl acceptors serving as precursors for guaiacyl and syringyl components, respectively, it remains still uncertain whether or not a single enzyme catalyzes the methylation of those hydroxycinnamic acids. Therefore, it is impotant to examine the possibility that the bamboo O-methyltransferase contain two different enzyme activities for the two natural substrates, CA and HF. This can be evaluated by measurement of varying ratios of the two enzymatically formed products during purification, as reported on phenylalanine- and tyrosine ammonia-lyases (16).

An attempt was made to separate O-methyltransferase activities for CA and HF using ammonium sulfate fractionation, isoelectric precipitation and DEAE-cellulose column procedures. A complete separation of these enzymes was never obtained. However, the ratio (SA/FA) varied after ammonium sulfate fractionations (Tables 5 and 6), which indicates the possible presence of two enzyme activities.

Iso-electric fractionations at pHs 4.5, 5.0 and 5.5 gave no significant variations of the ratio.

Table 5. CHANGES IN RATIO OF THE METHYLATED PRODUCTS AFTER AMMONIUM SULFATE FRACTIONATIONS OF THE ENZYME FROM Phyllostachys reticulata.

Enzyme	Incubation time in min	Products (n mole) FA	SA	Ratio (SA/FA)	mg protein
Buffer Ex.	60	55.0	60.0	1.09	62.5
0-40% $(NH_4)_2SO_4$ Fraction	0	0	0	-	4.0
	20	2.1	3.2	1.52	"
	45	4.9	8.3	1.70	"
	60	6.7	10.7	1.60	"
40-65% Fraction	30	60.0	59.4	0.99	14.3
	60	65.6	67.0	1.10	"
	90	62.0	56.4	0.91	"
65-80% Fraction	60	4.5	5.0	1.11	9.0

Stepwise elution on DEAE-cellulose gave neither significant changes in the ratio (Fig. 10 and Table 7). On the other hand, gradient elution on DEAE-cellulose gave two different activity curves (Fig. 11). The enzyme activity for CA shows three peaks; the greatest activity at No.65 and two lower activities at No.56 and 72. However, the enzyme activity for HF showed a quite different pattern; maximum activities were found at No.50, 61 and 72. The ratio also changed from 1.0 around No.40 to 0.2 at No.69. Therefore, the changes in the ratio, (SA/FA) after ammonium fractionations and the different elution patterns support the assumption that the bamboo O-methyltransferase contain at least two

different enzyme activities; one is involved in formation of guaiacyl lignins and the other, for formation of syringyl lignins. However, another possibility that each different enzyme itself also consists of a few iso-enzymes must be investigated. Because phenylalanine ammonia-lyase consists of two components (17,18). At any rate, further purification studies must be carried out in order to shed light on these complicated problems.

Table 6. CHANGES IN RATIO OF THE METHYLATED PRODUCTS AFTER AMMONIUM SULFATE FRACTIONATIONS OF THE ENZYME FROM Phyllostachys pubescens.

Enzyme	Incubation time in min	Products (n mole) FA	SA	Ratio (SA/FA)	mg protein
Buffer Ex.	20	125.0	119.0	1.0	16.5
Fraction I*	15	19.2	13.8	0.7	2.2
	30	26.4	26.0	1.0	"
Fraction II	15	34.2	39.0	1.1	2.5
	30	55.0	53.0	1.0	"
Fraction III	15	128.5	78.0	0.6	4.0
	30	167.0	90.0	0.5	"
Fraction IV	15	14.9	2.5	0.2	3.5
	30	21.0	6.9	0.3	"

*, Fraction I, 0-20% $(NH_4)_2SO_4$ precipitate; Fractions II, III, and IV are 20-35%, 35-55%, and 55-70% fractions, respectively. Assay conditions are described in the text (procedure 3).

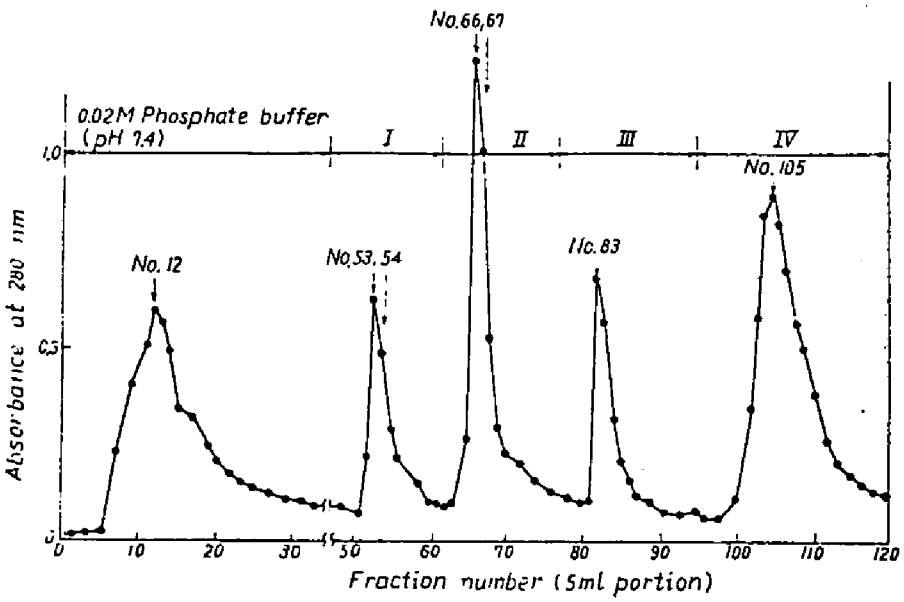

Fig. 10. STEPWISE ELUTION OF BAMBOO O-METHYLTRANSFERASE ON DEAE-CELLULOSE.

I, II, III and IV mean 0.02 M phosphate buffer solutions containing 0.1 M, 0.2 M, 0.3 M, and 0.6 M KCl, respectively. The numbers shown with arrows mean fractions employed for the enzyme assay.

Table 7. CHANGES IN RATIO OF THE METHYLATED PRODUCTS AFTER THE STEPWISE ELUTION THROUGH DEAE-CELLULOSE.

Fraction No.	Products (n mole) FA	SA	Ratio (SA/FA)
12	0	0	-
53	14.8	13.4	0.9
54	25.2	19.5	0.8
66	41.0	43.0	1.1
67	44.0	44.0	1.0
83	24.8	30.3	1.2
105	3.0	2.9	1.0

Assay conditions are described in the text.
Enzyme fraction precipitated between 40-60% $(NH_4)SO_4$ was used.

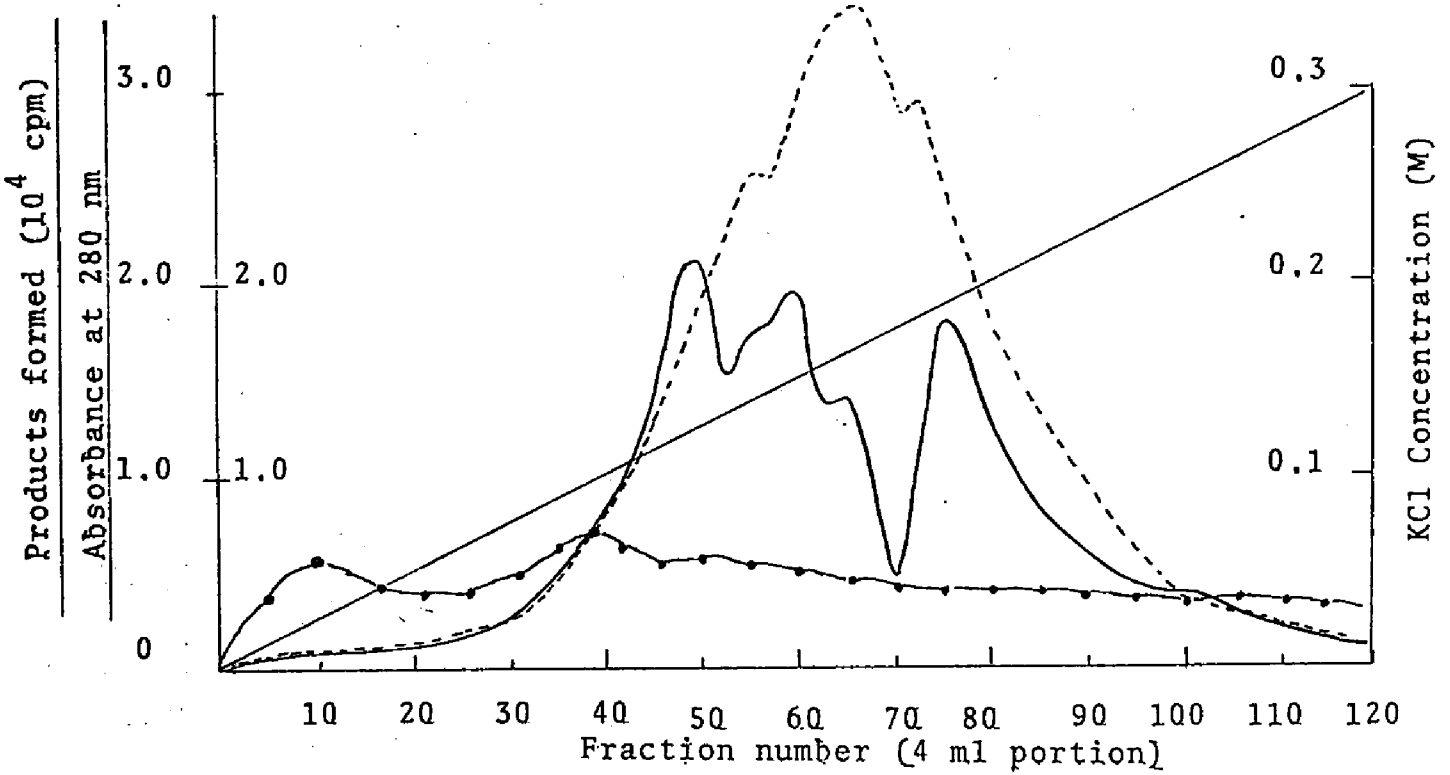

Fig. 11. GRADIENT ELUTION PATTERNS OF BAMBOO O-METHYLTRANSFERASE FROM *Phyllostachys pubescens* OBTAINED BY COLUMN CHROMATOGRAPHY ON DEAE CELLULOSE.

Assay conditions were described in the text, - - - - -, Enzyme activity for CA. ——, Enzyme activity for HF, —•—•—, Absorbance at 280 nm.

MATERIALS AND METHODS

Plant materials :

Fresh bamboo shoots of Phyllostachys pubescens (Mohso) and Phyllostachys reticulata (Madake) were used.

Reagents :

Cinnamic acid derivatives such as caffeic, ferulic, 5-hydroxyferulic, sinapic and 3,4,5-trihydroxycinnamic acids were synthesized from the corresponding bezaldehydes and malonic acid according to the method of Vorsatz (19) and Neish (20). Hydroxycinnamic acids-2-^{14}C synthesized in the same way using malonic acid-2-^{14}C procured from Japan Isotope Society. S-Adenosylmethionine iodide and its hydrogen sulfate were purchased from Calbiochemical Co. and Boeringer Mannheim Co., respectively. S-Adenosyl methionine-$^{14}CH_3$ was purchased from New England Nuclear, and methionine-$^{14}CH_3$ from Japan Radioisotope Society.

Extraction of the enzyme and assay of the activity:

1). All manipulations were carried out at 0-4° unless otherwise stated. The bamboo shoots were homogenized in a Waring blender with an equal weight of 0.1 M $NaHCO_3$ solution. The homogenate was filtered through gauze and the filtrate was centrifuged at 5,500xg for 20 min. To the supernatant solution was added solid $(NH_4)_2SO_4$ to 0.3 saturation with stirring. The resulting precipitate was centrifuged off and to the supernatant solution was added again $(NH_4)_2SO_4$ to 0.6 saturation. The collected precipitate was dissolved in 10 ml of 0.001 M potssium phosphate buffer (pH 7.0) and passed through a Sephadex G-25

column (2.8 x 30 cm) to remove $(NH_4)_2SO_4$. The eluate containing enzyme protein from the column was used for the assay of O-methyltransferase activity.

Reaction mixture contained following components ; 0.1 ml each of 0.01 M of CA, or HF (1 μmole), 0.01 M of $MgCl_2$ (1 μmole), 0.04 M sodium ascorbate (4 μmoles), 0.005 M S-adenosylmethionine iodide (0.5 μmole),0.2 ml of 1 M Tris buffer (200 μmoles, pH 8.0) and 1.4 ml of the enzyme solution.

The reaction mixture was incubated at 30° for 30 min and after addition of 0.5 ml of 10% HCl the reaction product was extracted with ether (3 x 5 ml portion). The ether was blown off by an electric fan, and the residue was submitted to paper chromatography (PPC) using toluene-AcOH-H_2O (4:1:5, upper layer)(descending method) as a solvent system. The separated fluorescent reaction products (FA or SA) were detected under u.v. light. The fluorescent band on the chromatogram was eluted with EtOH. FA or SA was determined by measurement of absorbance at 316 or 325 nm, respectively, using calibration curves previously made. These procedures were carried out quantitaively and the eluate from the paper of the same area in Rf value without containing fluorescence was always taken as a blank. The enzyme activity was expressed in the yield of ferulic acid (nano mole) per mg protein per 30 min unless otherwise stated.

The results obtained by this assay procedure are given in Figs. 1-7 and Tables 1-4.

2). The enzyme obtained in "procedure 1 " was found to lose the activity by addition of $(NH_4)_2SO_4$ or to be denatured by poly-

phenols. Therefore, in order to obtain the active enzyme in greater yield the extraction procedure employed in the " procedure 1 " was modified by addition of 2 mM NaN_3, EDTA, cysteine and iso-ascorbic acid and 0.1% bovine serum albumine into 0.1 M phosphate buffer solution (pH 7.5), with which plant tissue was homogenized. Other manipulations were the same as described above except that the original buffer extract was fractionated with $(NH_4)_2SO_4$ into 0-40, 40-65, and 65-80% fractions, successively.

The reaction mixture contained the following components; 0.1 ml each of 1 M Tris buffer (pH 8.0), 0.1 M $MgCl_2$, 0.1 M iso-ascorbate, S-adenosylmethionine sulfate (0.25 μmole), 0.2 ml of CA-2-^{14}C (0.5 μmole, 266 μCi/mM) or HF-2-^{14}C (0.5 μmole, 180 μCi/mM) and 1.0 ml of the enzyme solution. The reaction mixtures were incubated in duplicate for 30 min at 30° ; one test tube contained CA-2-^{14}C and the other, HF-2-^{14}C as a methyl acceptor. After the reaction was stopped by the addition of 0.5 ml of 10% HCl, 0.2 mg of non-labeled FA or SA was added as carriers into the respective reaction mixtures. Then, a pair of reaction mixtures were combined into one. The combined mixtures were extracted three times with 5 ml portion of ether. The ether was evaporated and the residue was submitted to PPC as described above. Two spots on the chromatogram containing FA and SA were separately cut into pieces and transferred into vial tubes, to each of which 1 ml of dioxane was added in order to elute the compound. After 30 min of incubation at room temperature, 10 ml of toluene scintillator solution containing 40 mg of PPO and 0.2 mg of POPOP was added and then

the radioactivities of FA and SA were determined with a Beckman scintillation counter. The enzymatically formed FA and SA were calculated from the radioactivities determined (counting efficiency, 85%). The results are given in Tables 1-7.

3). The extraction procedure was the same as in " procedure 2 " except that the original buffer extract was precipitated by addition of $(NH_4)_2SO_4$ to 0.8 saturation. The precipitated enzyme protein was stored at -20° until needed for experiments.

The reaction mixture contained the following components; 0.1 ml each of 1 M Tris buffer (pH 8.0), 0.1 M $MgCl_2$, 0.1 M NaN_3, 0.1 M iso-ascorbic acid, S-adenosylmethionine-$^{14}CH_3$(0.25 μmole, 0.05 μCi), 0.2 ml of non-labeled CA or HF (0.5 μmole), and 1.0 ml of the enzyme solution. The reaction mixture was incubated for 30 min at 30° . The products enzymatically metylated with $^{14}CH_3$ were extracted with ether (3 x 5 ml) after addition of 0.1 mg of cold FA or SA, separately. After evaporation of the ether, the residue was dissolved in 1.0 ml of dioxane and the radioactivities were determined by the scintillation counter as described above. The enzyme activity was expressed as nano mole of FA or SA formed which was calculated from the radioactivities obtained; 10^4 cpm corresponds to 26.6 n moles.

Stepwise elution column chromatography on DEAE cellulose :

Ammonium sulfate (40-65%) fraction was used for the experiment. After the enzyme protein through Sephadex G-25, the eluate (50 ml) containing 570 mg protein was applied onto the top of DEAE-cellulse column (1.6x13 cm). Unadsorbed proteins were washed down with 0.02 M phosphate buffer (pH 7.4)containing 1 mM

of cysteine. The adsorbed proteins were eluted stepwisely with the same buffer solutions containing 0.1 M, 0.2 M, 0.3 M, and 0.6 M KCl. The washings and the eluate were collected in 5-ml fractions following the absorbances at 280 nm with a UV-Auto (Ohtake). Several fractions with greater absorbances were chosen and dialyzed against 0.02 M phosphate buffer solution (pH 7.4) containing 1 mM of cysteine for 5 hrs at 0° . Then, O-methyltransferase activities of the fractions were assayed as described above. The enzyme was assayed by "procedure 2".

Gradient elution on DEAE-cellulose :

The enzyme protein obtained by "procedure 3" was used for this experiment. A portion of the enzyme was dissolved in 25 ml of 0.01 M phosphate buffer (pH 7.4) and centrifuged at 15,000 rpm for 20 min at 0° . The supernatant solution was dialyzed against 3 x 2.5 l of 0.01 M phosphate buffer solution containing 5 mM mercaptoethanol. The dialyzate containing 420 mg protein was applied onto a DEAE-cellulose column (1.1x10 cm) bufferized previously with the same buffer solution. After thoroughly washing the column, enzyme protein was eluted by a linear gradient method; a mixing chamber and a reservoir contained 250 ml of 0.01 M phosphate buffer (pH 7.4) and the same buffer with 0.3 M KCl, respectively. The enzyme activity was assayed on every second fraction by use of 0.5 ml from each fraction according to "procedure 3".

Extraction of " methionine activating enzyme " :

Fresh bamboo shoots were homogenized with large amount of cold acetone (-20°) and the homogenate was immediately filter-

ed. The acetone powder thus obtained was thoroughly dried in vacuo in a desiccator.

The reaction mixture contained the following components; 0.1 ml each of CA (10 μmoles), $MgCl_2$ (10 μmoles), ATP (20 μ moles), KCl (20 μmoles), glutathione (20 μmoles), methionine-$^{14}CH_3$ (0.2 μCi, 6.3 mCi/mM), cold methionine (20 μmoles) and 0.2 ml of 2 M Tris buffer (pH 7.5) and 100 mg of the acetone powder. The reaction mixture was incubated at 30° for 5 hrs, then, the reaction was stopped by addition of 0.5 ml of 10% HCl. Enzymatically formed radioactive FA and S-adenosylmethionine were identified as follows ; The mixture was extracted with ether. The ether was evaporated and the residue was submitted to PPC with toluene-AcOH-H_2O (4:1:5). The water phase was centrifuged and the supernatant was applied onto a Dowx 1x8 column (Cl form) after neutralization of the supernatant. S-Adenosylmethionine eluted with water was followed by measurement of absorbance at 260 nm. The eluate containing S-adenosylmethionine was evaporated and the residue was submitted to PPC with n-BuOH-AcOH-H_2O (5:1:4) (21). The radio active FA and S-adenosylmethionine on the chromatograms were scanned with a radiochromatogram scanner (Aloka PCS-4).

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CHAPTER III

CHANGES IN ACTIVITIES OF THE ENZYMES AND IN METABOLISM OF PRECURSORS IN RELATION TO BAMBOO LIGNIN FORMATION

INTRODUCTION

Several enzymes involved in biosynthesis of lignins were demonstrated to be present in growing bamboo shoots (1-6) and their properties were described in Chapter II.

Concerning control mechanism in lignin formation it is interesting to know how those enzymes work and how the lignin precursors are metabolized during lignification of plants. However, such investigations on the enzymes in woody plants are very few and systematic investigations have been expected in relation to lignin formation. For such studies growing bamboo shoots are excellent plant materials because during growth of bamboo shoots the changes in activities of the enzymes and in metabolism of intermediates accompany the successive stages of lignification in a single maturing plant. In addition, the shoots are very suitable as an enzyme source, since these monocotyledoneous plants yield no browning matters on homogenization, whereas other woody plants give intensive browning which often denatures enzymes.

The present Chapter, in relation to the lignification of bamboo, describes the changes in the activities of the enzymes involved in pentose phosphate, shikimate, and cinnamate pathways.

RESULTS AND DISCUSSION

The enzymes in pentose phosphate pathway:

Embden-Meyerhof-Parnas and pentose phosphate pathways are well known as the two main pathways of sugar metabolism in higher plants. Pentose phosphate pathway contributes not only to the formation of $NADPH_2$ but also to the formation of shikimic acid which is synthesized by condensation of erythrose-4-phosphate and phosphoenolpyruvate.

As shown in Fig. 1, the activities of G-6-P and 6-PG dehydrogenases seem to be activated and maintained to a certain level toward the basal parts of the bamboo shoots. These patterns of the activities indicate that both enzymes are correlated with the lignification. However, with a larger bamboo shoots (4-5 m in height) sampled before shooting of the branches, the enzyme activities were highest in the apex and decreased toward the lower parts of the shoots.

In order to obtain information about the relative extents of utilization of glucose in the two pathways in growing bamboo, the "C_6/C_1 ratio" was measued by supplying the tissues with glucose-6-^{14}C and glucose-1-^{14}C. Fig. 2 shows that total amount of O_2-uptake by tissues decreased and C_6/C_1 ratio also declined toward the lower parts. In view of the fact that the C_6/C_1 ratio obtained with mature tissues was lower than that with young meristematic tissues (7-9), the above-described results may be taken as suggestive of the domination of the pentose phosphate pathway in the respiratory process of lignifying tissues. Similar results were obtained with tissues of outermost sapwood adjacent to cambial zones of trees (10).

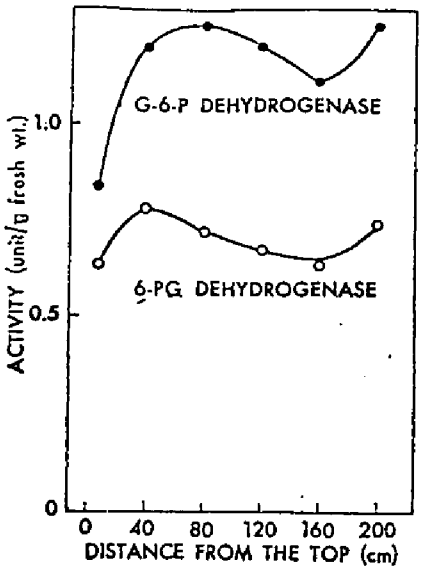


Fig. 1. CHANGES IN ACTIVITIES OF G-6-P AND 6-PG DEHYDROGENASES IN DIFFERENT PARTS OF BAMBOO SHOOT.

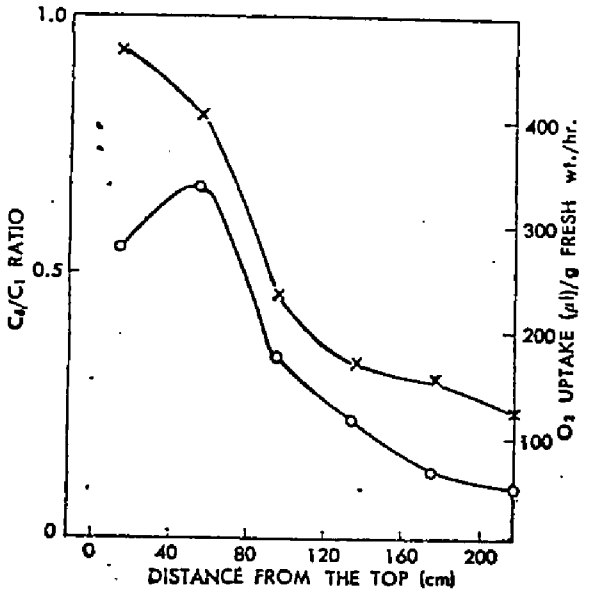


Fig. 2. PATTERN OF RESPIRATORY BREAKDOWN OF LABELED GLUCOSE.
-o-, C_6/C_1 ratio,
-x-, O_2-uptake (µl).

Shikimic acid and the enzymes in shikimate pathway:

Since the first evidence for the operation of the shikimic acid pathway in lignin formation was provided by Brown and Neish (11), it is established that shikimic acid plays an important role as a precursor of naturally occurring aromatic amino acids, flavonoids (12) and lignins (13-14) in higher plants. Therefore, variation of shikimic acid content and

changes in activities of 5-dehydroquinate hydro-lyase and 5-dehydroshikimate reductase (shikimate:NADP oxidoreductase) were examined with growing bamboo shoots.

As shown in Figs. 3 and 4, 5-dehydroquinate hydro-lyase and 5-dehydroshikimate reductase exhibited maximum activity at the locus immediately below the apex and a quite gradual decline in activity toward the basal parts of the shoots. The shikimic acid content of the bamboo tissue had a pronounced maximum below the apex as shown in Fig. 5. It appears that young tissues synthesize shikimic acid vigorously as a precursor for phenylalanine and tyrosine that are incorporated into protein. Consequently, the increase in the acid content just below the apex and the decrease in the basal parts show that shikimic acid is, in fact, a metabolically active compound in a growing bamboo, which is in good agreement with the early findings.

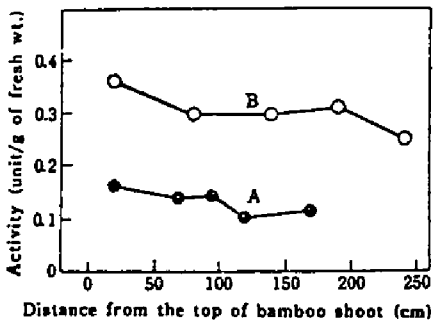


Fig. 3. ACTIVITIES OF 5-DEHYDROQUINATE HYDRO-LYASE IN DIFFERENT PARTS OF BAMBOO. A; Sampled on May 25th, 2.5 m in height. B; Sampled on May 29th, 4.0 m in height.

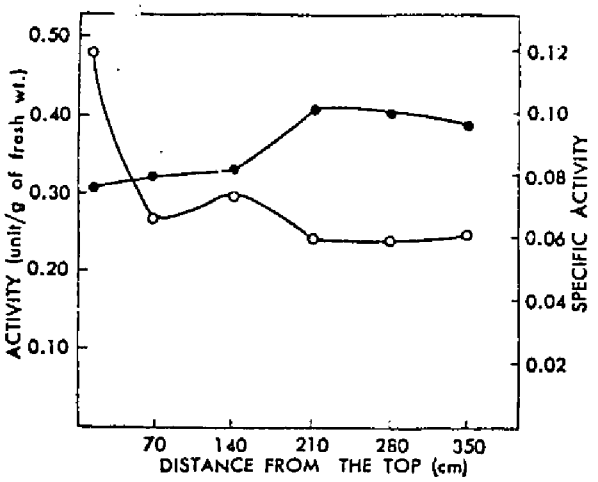


Fig. 4. ACTIVITIES OF 5-DEHYDRO-SHIKIMATE REDUCTASE IN DIFFERENT PARTS OF BAMBOO SHOOT.
—o—, Activity(unit/fr,wt.)
—•—, Specific activity (unit/mg protein)

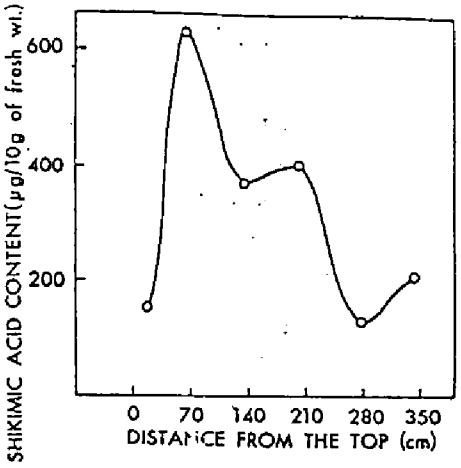


Fig. 5. VARIATION OF SHIKIMIC ACID CONTENT IN DIFFERENT PARTS OF BAMBOO SHOOT.

The activities of 5-dehydroquinate hydro-lyase and dehydro-shikimate reductase were found to increase by wounding (15) or light irradiation of plant tissues (16). However, such a phenomenon was not observed in the growing bamboo shoots.

The precursors and the enzymes in phenylalanine-cinnamate pathway:

Phenylalanine has been known to be a natural intermedate of phenylpropane constituents of lignins in higher plants (17). Tyrosine has also been shown to be incorporated into lignins of a few families of higher plants such as Gramineae and the Compositae (18, 19). These two aromatic amino acids are formed by mediation of transaminase (20) from phenylpyruvic and p-hydroxyphenylpyruvic acids, respectively and converted to cinnamic and p-coumaric acids, by phenylalanine- (21) and tyrosine ammonia-lyases (22), respectively.

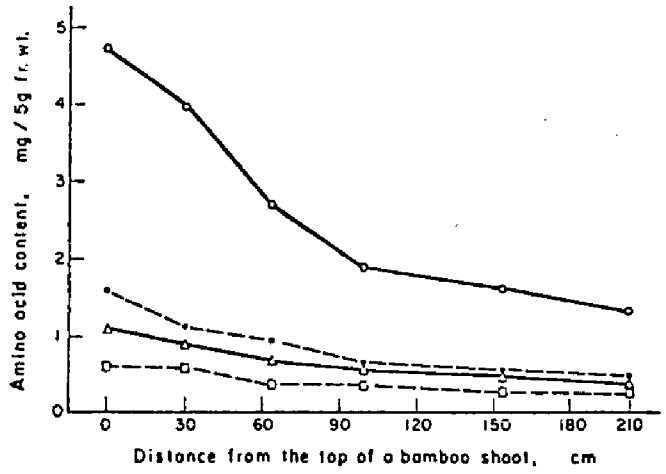


Fig. 6. CHANGES IN AMINO ACID CONTENT WITH GROWTH OF A BAMBOO.
—○—, tyrosine; --●--, phenylalanine;
—△—, methionine; --□--, glutamic acid

The free amino acids contained in a young bamboo were examined by paper chromatography and the following were detected: phenylalanine, tyrosine, alanine, leucine, methionine, valine, proline, lysine, serine, glutamic acid, tryptophan, aspartic acid etc. in good accordance with results already reported (23).

Among these amino acids, phenylalanine, tyrosine, methionine, and glutamic acid, which are probably all involved in the formation of lignin precursors, were examined further. Both phenylalanine and tyrosine are efficiently utilized as precursors for lignins in grasses; methionine is known to be a methyl donor for the methoxyl groups of lignins (24), glutamic acid, which is a main amino donor in transamination reactions in plant tissues, was selected for measurement, although it may not be directly related to lignin formation.

Figure 6 shows the changes in contents of those four amino

acids during the growth of a bamboo. Tyrosine was present in the highest amount at any stage of growth, and was highest in the tissue at the apex of the shoot, decreasing rapidly toward lower parts of the shoot. The other acids were present in much smaller amounts and showed less drastic decreases during the growth. The decrease in tyrosine content is very indicative of the presence of a system metabolizing tyrosine efficiently, which is in good agreement with the increase in tyrosine ammonia-lyase during lignification of a growing bamboo (25).

Attempts were made to isolate a transaminase from bamboo shoots, but reproducible results could not be obtained. Sliced tissue of the bamboo shoot was therefore used for the estimation of transaminase activity with phenylpyruvate and p-hydroxyphenylpyruvate as substrates. It was found that phenylalnine and tyrosine were formed in substantial amounts by incubation of the substrates with the sliced tissue. p-Coumaric acid was also found to be formed and isolated from both incubated reaction mixtures (5). Since it is impossible to express the transaminase activities in the amounts of the two aromatic amino acids formed, the activities were, for the time being , determined as the recovered amounts of the substrates after the incubation. The changes in transaminase activities during the growth of a bamboo are given in Fig. 7. The results indicate that more phenyl pyruvate and p-hydroxyphenylpyruvate was recovered from the reaction mixtures when tissue slices from the upper part of the plant were used, suggesting that the tissue at the apex has a lower metabolic activity than the tissue from lower parts of the shoot.

The patterns of the formation of phenylalanine and tyrosine were determined in the same series of experiments (Fig. 8). The results obtained appear somewhat similar to those shown in Fig. 6. The fact that higher amounts of phenylalanine and tyrosine were obtained from the upper tissues indicates that these tissues cannot convert phenylalanine and tyrosine to other substances, such as cinnamic acids, as rapidly as can lower tissue. The changes in the formation of p-coumaric acid from phenylpyruvate and p-hydroxyphenylpyruvate seem to support this explanation (Fig. 9). Because p-coumaric acid, formed via the corresponding aromatic amino acids, was accumulated in higher amounts with tissues from the lower parts.

Metabolic studies with the tissue samples from various parts of the shoot showed that the activity of transaminase increased toward the lower parts of the shoot. The results further suggest that the para-hydroxylation to p-coumaric acid from cinnamic acid formed by deamination of phenylalanine is mediated in the tissues. Cinnamic acid-4-hydroxylase (26-28) is presumed to be functioning intensively because higher amounts of p-coumaric acid were obtained from the reaction mixtures with phenylpyruvate as a substrate. The possibility of hydroxylation of phenylalanine to tyrosine was ruled out in the present experiment by the negative results obtained from tracer experiments with labeled phenylalanine-U-^{14}C, although Nair and Vining[26] demonstrated phenylalanine hydroxylase in spinach leaves.

From the results of present experiments and the results reported by Gamborg and Wetter (20), aromatic amino acid transami-

nase may contribute to the formation of lignin precursors by supplying phenylalanine as a substrate.

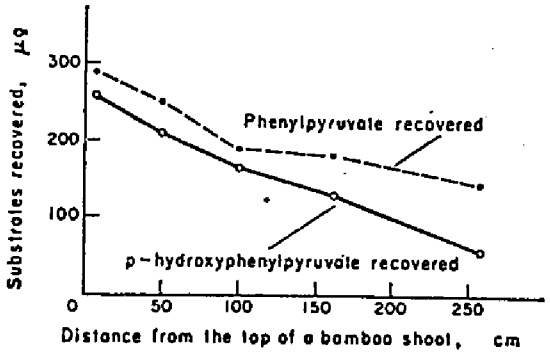


Fig. 7. CONSUMPTION PATTERN OF PHENYLPYRUVATE AND p-HYDROXY-PHENYLPYRUVATE INCUBATED WITH SLICED TISSUE.

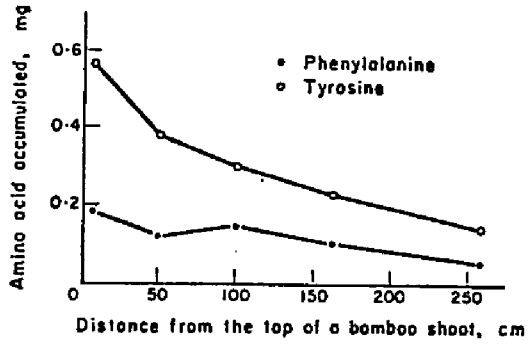


Fig. 8. ACCUMULATION OF THE AROMATIC AMINO ACIDS BY THE TISSUE.

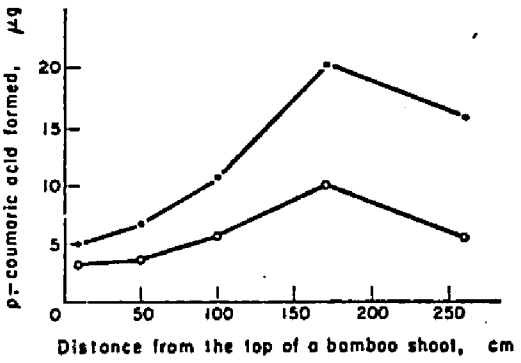


Fig. 9. FORMATION OF p-COUMARIC ACID BY SLICED TISSUE.
—●—, p-coumaric acid formed from phenylpyruvate;
—○—, the acid formed from p-hydroxyphenylpyruvate.

Furthermore, the fact that p-hydroxyphenylpyruvate was converted to both tyrosine and p-coumaric acid, acting as lignin precursors, in bamboo shoots is in good agreement with the results obtained by Acerbo, Schubert and Nord (29) and by Wright and Neish (30). Therefore, the conversion of the phenylalanine and tyrosine to lignins is of specific interest from the point of view of lignin biosynthesis. Phenylalanine ammonia-lyase (phenylalanine deaminase, PAL) was first isolated from acetone powders of barley by Koukol and Conn (21). Neish (22) found tyrosine ammonia-lyase (tyrase, TAL) in all members of Graminaceae that were studied such as sorghum, wheat, corn, barley, oats, rice and sugar cane. The enzyme could not be detected in peas, lupine or sweet clover (22) or in tissue cultures of conifers and woody angiosperms (31-32).

Bamboo and grass lignins differ from lignins in other higher plants by virtue of their high content of p-coumaric acid ester groups (33-34) and there exists a probable connection between these groups and the presence of TAL. It is possible as proposed by Higuchi (25) that a large portion of p-coumaric acid generated through the deamination of tyrosine becomes channeled to these ester groups.

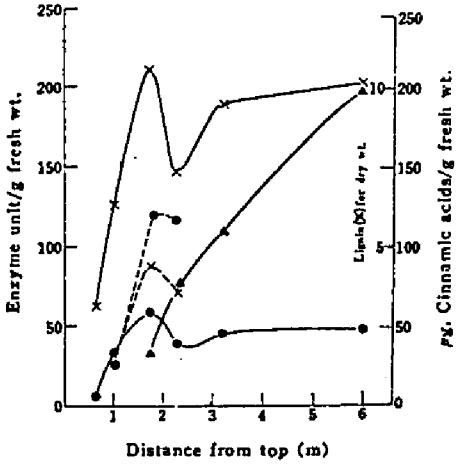
The changes in activities of the two ammonia-lyases (Fig. 10), cinnamic acid-4-hydroxylase (Fig. 11) and O-methyltransferase (Fig. 12) were investigated in relation to lignin formation of bamboo shoots. As shown in Fig. 10, the fact that the activities of PAL and TAL increased from the apex toward the basal parts of the shoot, i.e. with proceeding of lignification, coincides well with the fact that the pool sizes of the aromatic

amino acids became smaller toward the lower parts of the shoots (Fig. 8), indicating that the two enzymes are intimately correlated with lignin formation. The same results were already reported with buck wheat by Yoshida and Shimokoriyama (35). A similar relationship between the rate of lignification and PAL was established for Sequoia by Rubery and Northcote (36).

Cinnamic acid-4-hydroxylase also increased toward the lower parts of the shoot. Therefore, it may be considered that this enzyme is not only related with lignin formation but also plays an important role in formation of p-coumaric acid (Tables 1 and 2) and its esters associated with lignin (Fig.13).

O-Methyltransferase also showed the patterns of increase in activity during the growth of bamboo shoots(Fig. 12), which means that the enzyme contributes to the formation of guaiacyl and syringyl lignin components by methylating caffeic and 5-hydroxyferulic acids, respectively, during the lignification.

Since it was found that bamboo O-methyltransferase can utilize both caffeic and 5-hydroxyferulic acids as natural substrates (see Chapter II. 4), changes in the ratio of two enzyme activities which were expressed as the ratio of the respective two products, (SA/FA) were examined using the bamboo shoots with various growth stages. The results are given in Table 3. The aim of this examination was to detect two different activities of O-methyltransferase during growth of the shoots. Because the ratio, S/V (syringaldehyde/vanillin) increased with the growth of bamboo shoots (37). However, the expected result was not obtained because the ratio did not change but was one for every tissue sample tested. Therefore,



-x-, phenylalanine deaminase
-o-, tyrase
--x--, cinnamic acid
--o--, p-coumaric acid
-▲-, lignin

Fig. 10. CHANGES IN ACTIVITIES OF THE ENZYMES, CINNAMIC ACID CONTENTS, AND LIGNINS CONTENT OF DIFFERENT PARTS OF AN IMMATURE BAMBOO (P. pubescens, 8.8 m in length). This figure was cited from Ref.(25).

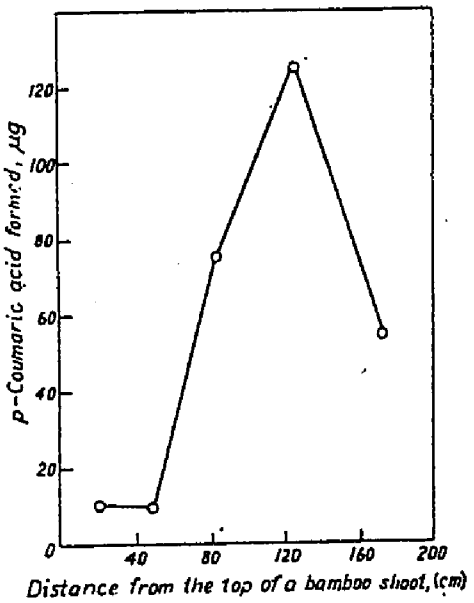


Fig. 11. PATTERN OF CINNAMIC ACID HYDRXYLASE ACTIVITY OF DIFFERENT PARTS OF THE SHOOT (P. pubescens).

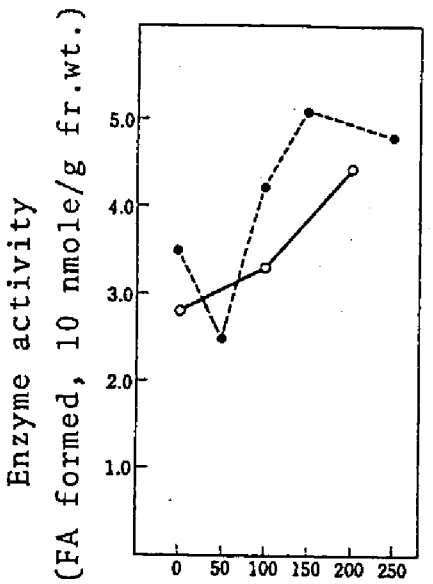


Fig. 12. CHANGES IN ACTIVITY OF O-METHYLTRANSFERASE.

P. reticulata was used for the enzyme assay.

—○—; bamboo shoot, 220 cm in length.

—●—; bamboo shoot, 280 cm in length.

TABLE 1. VARIATIONS IN THE CONTENT OF FREE *p*-COUMARIC AND FERULIC ACIDS IN BAMBOO (*Phyllostachys pubescens*) WITH GROWTH

Distance from the top (cm)	*p*-Coumaric acid (μg/10 g fresh weight)	Ferulic acid (μg/10 g fresh weight)
0– 20	2·6	0·2
80– 90	2·9	1·1
140–150	10·6	3·1
190–200	55·6	3·8
260–270	248·8	4·5
340–350	398·7	1·7

TABLE 2. VARIATIONS IN CONTENT OF FREE *p*-COUMARIC AND FERULIC ACIDS WITH GROWTH OF A BAMBOO (*Phyllostachys reticulata*)

Distance from the top (cm)	*p*-Coumaric acid (μg/10 g fresh weight)	Ferulic acid (μg/10 g fresh weight)
0– 10	2·7	2·0
40– 50	5·0	2·1
90–100	55·5	3·9
120–130	152·8	5·2
170–180	116·8	5·5
210–220	161·6	7·3

Table 3. CHANGES IN THE RATIO (SA/FA) DURING GROWTH OF BAMBOO SHOOTS (Phyllostachys pubescens).

Sample**		Products (n mole)* FA	SA	Ratio (SA/FA)
	A***	60	55	0.9
A.	M	85	74	0.9
	L	82	78	1.0
	A	98	104	1.1
B.	M	84	88	1.0
	L	69	77	1.1
	A	84	72	0.9
C.	M	108	96	0.9
	L	92	109	1.2
	A	60	60	1.0
D.	M	73	73	1.0
	L	43	43	1.0
	A	125	119	1.0
E.	M	230	231	1.0
	L	239	248	1.0

*, S-Adenosylmethionine-$^{14}CH_3$ was used as a methyl donor. The assay conditions were described in the text.

**, Samples, A,B,C,D and E; 15, 20, 50, 50, and 270 cm in length, respectively.

***, A; apical part, M; middle part, L; lower part.

it may be considered that the two activities exist in an equal amount throughout the growth of the shoots, on the assumption that O-methyltransferase consists of two different enzymes for guaiacyl and syringyl components (See Chapter II,4).

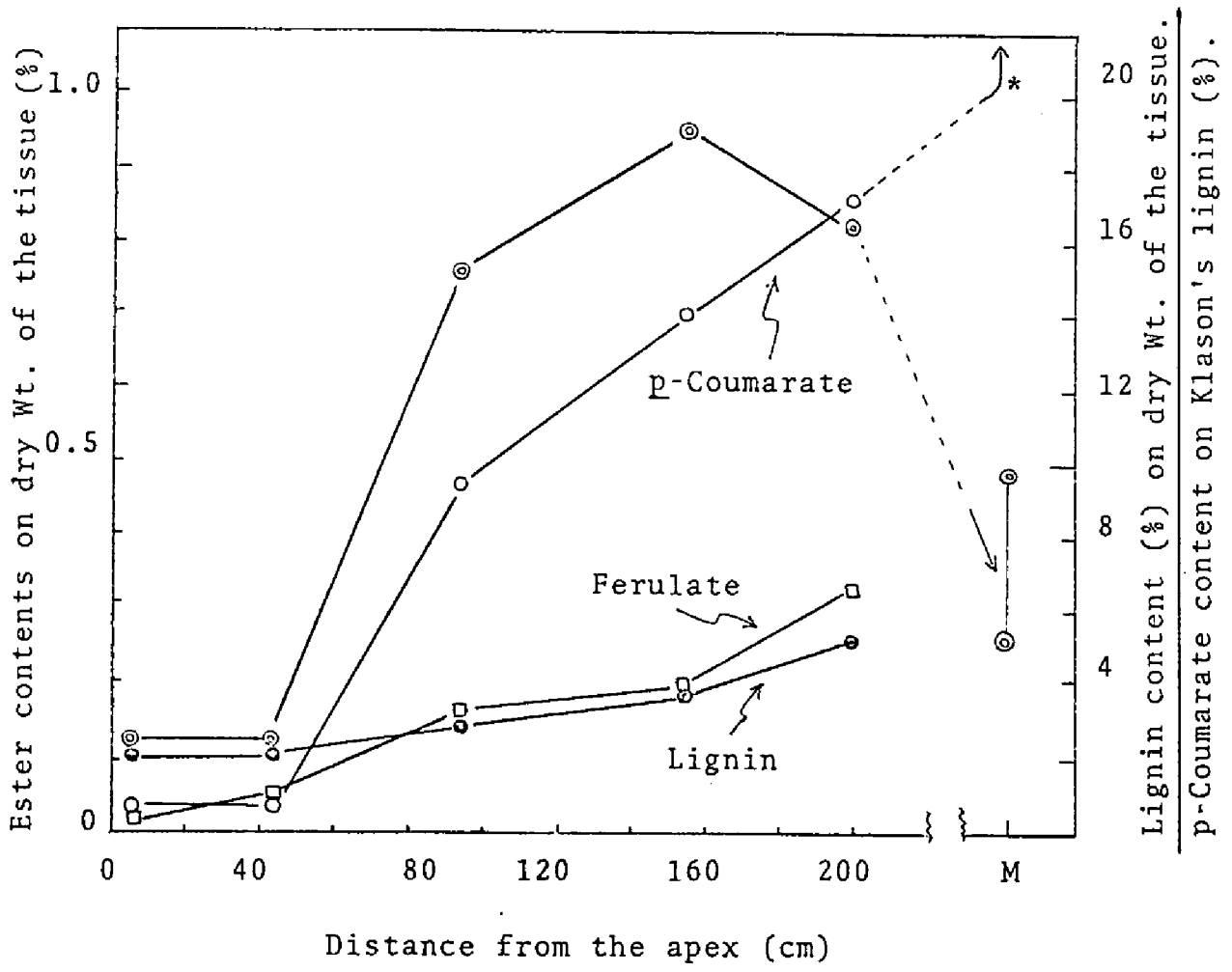


Fig. 13 CHANGES IN CONTENTS OF HYDROXYCINNAMIC ACID ESTERS AND LIGNIN (Phyllostachys reticulata).

M; Mature bamboo, *; p-coumarate ester content (2-3%) on dry Wt. of mature bamboo. -◎-, p-coumarate content based on lignin.

As described above, the activities of the enzymes involved in the transamination, the deamination, the hydroxylation and O-methylation steps were found to be intimately correlated with the lignification of bamboo shoots whereas the enzymes in pentose phosphate and shikimate pathways did not show such marked increasing patterns in the activities. Similarly, the enzymes in TCA cycle did not increase but rather decreased during lignification (5). From these results it is reasonable to consider

that the enzymes in the phenylalanine-cinnamate pathway situated at the locus quite near to the end product of lignin are activated rather than the enzymes in pentose phosphate and shikimate pathways situated at the early stages in the metabolic pathway leading to lignin.

In connection with the changes in enzyme activities, it is of interest to investigate behaviors of the esters of p-coumaric and ferulic acids that are demonstrated to be natural precursors of lignins (38-41). The intensive accumulation of free p-coumaric and ferulic acids are already shown in Tables 1 and 2. Similar accumulation patterns for p-coumarate and ferulate esters bound to cell wall constituents are given in Fig 13. Fig. 14 shows relationships between p-coumarate and lignin, and ferulate and carbohydrate, indicating that p-coumarate ester groups are exclusively bound to lignin molecules, whereas ferulate ester groups are to carbohydrate molecules. Therefore, it can be considered that the increase in the amounts of p-coumarate ester during the growth of the shoots is more correlated with lignin formation than that of ferulate. This explanation is supported also by the fact that bamboo milled wood lignin contains 5 to 10% of p-coumarate ester accompanied by ferulate in a small amount (33-34).

More interesting is the fact that the relative content of p-coumarate ester based on Klason's lignin increased from the apex, reaching a maximum at the locus around 150 cm and finally decreasing to the content (5-10%) for mature bamboo as shown by a dotted line in Fig. 14. On the other hand, the ester content on dry weight of the shoot increased intensively

from the apex to the basal parts, finally reaching the greatest amount (2-3%) as shown by another dotted line in the Fig. This indicates that the greater majority of p-coumarate ester groups are formed at the early stages of lignification, since the content of the ester in the young bamboo shoot was 2 to 3 times greater than that of the ester in the grown-up one. Accordingly, it may be assumed that p-coumarate ester groups are heterogeneously distributed within cell wall, although merely average content of the ester was obtained by the conventional method of milled wood lignin preparation. The ester linkages of p-coumarate is discussed in Chapter VI (42).

Metabolic regulation of lignification :

There are many factors influencing lignification. Siegel and his coworkers reported that IAA repressed lignin formation, indicating that IAA acts as an antioxidant to inhibit the oxidation of phenolic compounds catalyzed by peroxidase (43). Koblitz recognized that kinetin increased lignin content from 20 to 30% in carrot tissues (44). Bergman (45) also obtained the similar results that lignin content in tobacco tissues grown under the presence of kinetin increased from 4 to 22%, suggesting that this hormone could activate the enzymes in the pentose phosphate, the shikimate and the cinnamate pathways. Gibberellins are also considered to promote the lignin formation (46-47). However, the physiological nature of the trigger for lignification is still obscure. The results described above on the variations of the enzyme activities point out that from a physiological aspect, activation or synthesis of the enzymes in phenylalanine-

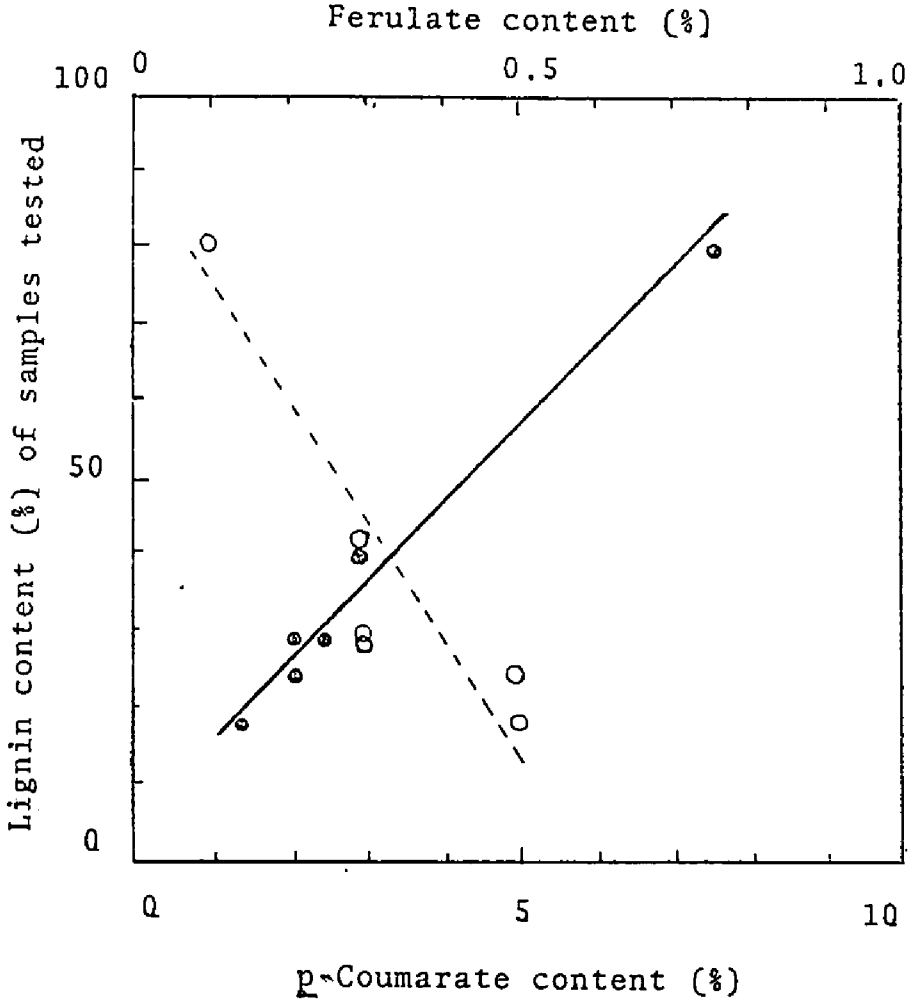


Fig. 14. THE RELATIONSHIPS BETWEEN LIGNIN CONTENT AND p-COUMARATE AND FERULATE ESTERS CONTAINED IN MWL AND LCC FRACTIONS FROM BAMBOO MEAL (P. pubescens).

-●-, p-coumarate; --○--, ferulate.
Lignin content was determined by the acetyl bromide method and expressed as Klason's lignin. The method of preparation of MWL and various LCC fraction is described in the text.

cinnamate pathway, e.g. the activation of PAL, is one of the most important factors to induce lignin formation. Because PAL is located at a diverging point on the metabolic pathways for "primary metabolites" (amino acid and protein) and "secondary metabolites" (phenolics including lignin). Zucker (48) reported

that PAL in the potato tuber was induced by light irradiation although no PAL activity could be detected in the fresh tissue before the irradiation. Yoshida also reported that the PAL synthesis was greatly enhanced by light in germinating pea seedlings (49), indicating that phytochrome and the related pigments are involved as an inducer in the enzyme appearance. A similar inducer may participate in the formation of PAL in growing bamboo shoots.

As another regulating factor for lignification, NADP level in tissues should be considered. As described above, the enzymes in pentose phosphate and shikimate pathways are NADP-specific and accordingly low level of NADP should repress the function of these enzymes. As factors to increase NADP level in plant tissues, oxygen, red light and kinetin have been known (50). Although such many factors are involved in initiation of lignification, further investigations are needed on regulating mechanism for induction of enzyme synthesis during plant development.

MATERIALS AND METHODS

Assay of the enzymes in pentose phosphate pathway:

Extraction and assay method are described in Chapter II-1. For comparison of the enzyme activities in different parts of bamboo shoot, *P. reticulata* was used, because in the course of this experiment immature Mohso (*P. pubescens*) was out of season. An immature bamboo (2.4 m in length) was cut into 6 pieces at about equal intervals. From each piece 20 g of tissue was cut out and homogenized with 40 ml of 0.1 M $NaHCO_3$ at 0~4° . The homogenate was used as an enzyme preparation after centrifugation.

The enzyme activities of 6 different preparations were expressed on g fresh wt. for comparison.

Respiratory breakdown of G-1-^{14}C and G-6-^{14}C :

Fresh immature shoot of Mohso (P. pubescens) 2.4 m in length was cut into 6 pieces at about equal intervals. From each piece two sliced sections (500 mg for each) were cut out. One was used for respiration experiment with glucose-1-^{14}C and the other with glucose-6-^{14}C. Oxygen uptake was measured by using Warburg respirometer at 30°. In a main compartment 500 mg of tissue, 2.0 ml of 0.1 M sodium phosphate buffer (pH 6.8) and 0.5 ml of specifically labeled glucose (0.1 μCi) were added and in a center well 0.2 ml of 10% potassium hydroxide. The radioactive carbon dioxide liberated for 4 hr in respiratory breakdown of the labeled glucose was converted to barium carbonate and the radioactivity was measured by a gas flow counter, and the C_6/C_1 ratio was calculated.

Extraction and assay of the enzymes in shikimate pathway:

The extraction and assay conditions of 5-dehydroquinate hydrolyase and dehydroshikimic reductase were described in Chapter II-2 and Chapter II-3, respectively. However, for the comparison of the activity of the enzyme in different parts of bamboo shoot, the following preparation was used. Bamboo shoot about 2.5-4.0 m in length was cut into five parts at a suitable interval. From each part, constant weight of tissue was cut out into small pieces and homogenized with equal weight of 0.05 M potassium phosphate buffer (pH 7.4) with an Ultra turrax homogenizer. The homogenate was squeezed through gauze, the filtrate

was centrifuged at 10,000xg for 20 min. and to the supernatant solution solid ammonium sulfate was added to 0.70 saturation. the precipitate collected by centrifugation was dissolved in 0.05 M potassium phosphate buffer (pH 7.4). Different enzyme preparations thus obtained were used for the assay of 5-dehydroquinate hydro-lyase.(See Ref.2).

In a similar way the assay of dehydroshikimate reductase was carried out and each enzyme activity was compared (3).

Determination of shikimic acid:

Shikimic acid was isolated from different parts of bamboo and determined according to the method of Yoshida and Hasegawa (51). The immature shoot of Mohso 4.2 m in length was cut into 6 pieces at about equal intervals and from each portion 10 g of tissue was cut out. Each piece was homogenized and extracted with hot ethanol for several minutes. The extract was filtered and evaporated in vacuo and the residue was dissolved in water (30 ml). The solution was passed through a column of anion exchanger (Amberlite 410). Shikimic acid adsorbed on the anion exchanger was eluted with 1 M ammonium carbonate and the effluent was evaporated to dryness and the residue was dissolved in 5.0 ml of water. The solutions containing shikimic acid were subjected to colorimetric determination(51).

Determination of free amino acids:

Young bamboo (P. pubescens), 2 to 3 m in height was used for determination of free amino acids. Fresh tissue was cut from six parts from the apex to the base of the shoot. 10 g of each was homogenized in hot 70% EtOH(100 ml) and the filtrate

and washings were evaporated *in vacuo* at about 50°.
The amino acids were quantitatively determined by two-dimensional paper chromatography.

Metabolic activities of bamboo tissue for transamination (Fig.7-9):

(a) 5 g of sliced tissue from young bamboos (*P. reticulata*) was infiltrated *in vacuo* with a solution of sodium phenylpyruvate (or *p*-hydroxyphenylpyruvate, 5 μmoles), glutamic acid (5 μmoles), sodium ascorbate (25 μmoles) in H_2O (1.0 ml). These 6 incubation mixtures were kept at 25° and after homogenization of the incubated tissues the products (phenylalanine and tyrosine) were determined.

(b) p-Coumaric acid formed from phenylpyruvate or from *p*-hydroxyphenylpyruvate was also determined in the test solutions used for the estimation of amino acids. The compound was isolated by PPC with toluene-AcOH-H_2O(4:1:5,V/V, organic layer) for 10 hr. The *p*-coumaric acid spot on the chromatogram was then cut out and eluted with 95% EtOH and its absorptivity measured at 310 nm.

(c) A young bamboo (*P. reticulata*), about 3.0 m in height, was used for the experiments on changes in the enzyme activities. Five samples of the tissues were prepared from sections 5, 50, 100, 170 and 260 cm from the apex of the shoot. The sliced samples (5 g) were infiltrated with substrates and the reaction systems were incubated for 90 min at 25° together with controls minus substrates. The solution obtained, after EtOH-extraction, were used for the determination of phenylalanine, tyrosine and p-coumaric acid formed as described above.

Phenylpyruvate and p-hydroxyphenylpyruvate as substrates were recovered and determined by taking the absorbance of the EtOH-extracts in 0.1 N NaOH at 320 and 330 nm, respectively, against a blank.

Changes in cinnamic acid hydroxylating activity:

From each of five different parts of the bamboo shoot (P. pubescens) 2 m in height, 5 g of tissue was cut out, sliced and incubated with 0.5 mg of the labeled cinnamic acid at 25° for 5 hr. Net synthesis of p-coumaric acid was determined as described above (Fig. 11).

Changes in O-methyltransferase activities:

The activities of O-methyltransferase extracted from different parts of bamboo shoots were assayed and compared with one another. The assay conditions were the same as described in Chapter II-4,(Procedure 1). (Fig.12).

Determination of free p-coumaric and ferulic acids:

Young bamboos, 2.5-3.6 m in height, were cut and 10 g of fresh tissue taken from six different parts of the shoot, and homogenized with the Ultra-turrax homogenizer with about 50 ml of hot 80% EtOH. The homogenate was filtered through a celite-bedded glass filter and washed with EtOH. The concentrate was dissolved in 40 ml of hot H_2O, again filtered and the filtrate and washings were extracted with Et_2O and after acidifying the aqueous solution with 10% HCl, it was extracted with ether. The ether extract, after washing with water, was dried (Na_2SO_4) and evaporated to dryness. The concentrate was submitted to PPC with toluene-AcOH-H_2O(4:1:5,V/V). The spots corresponding

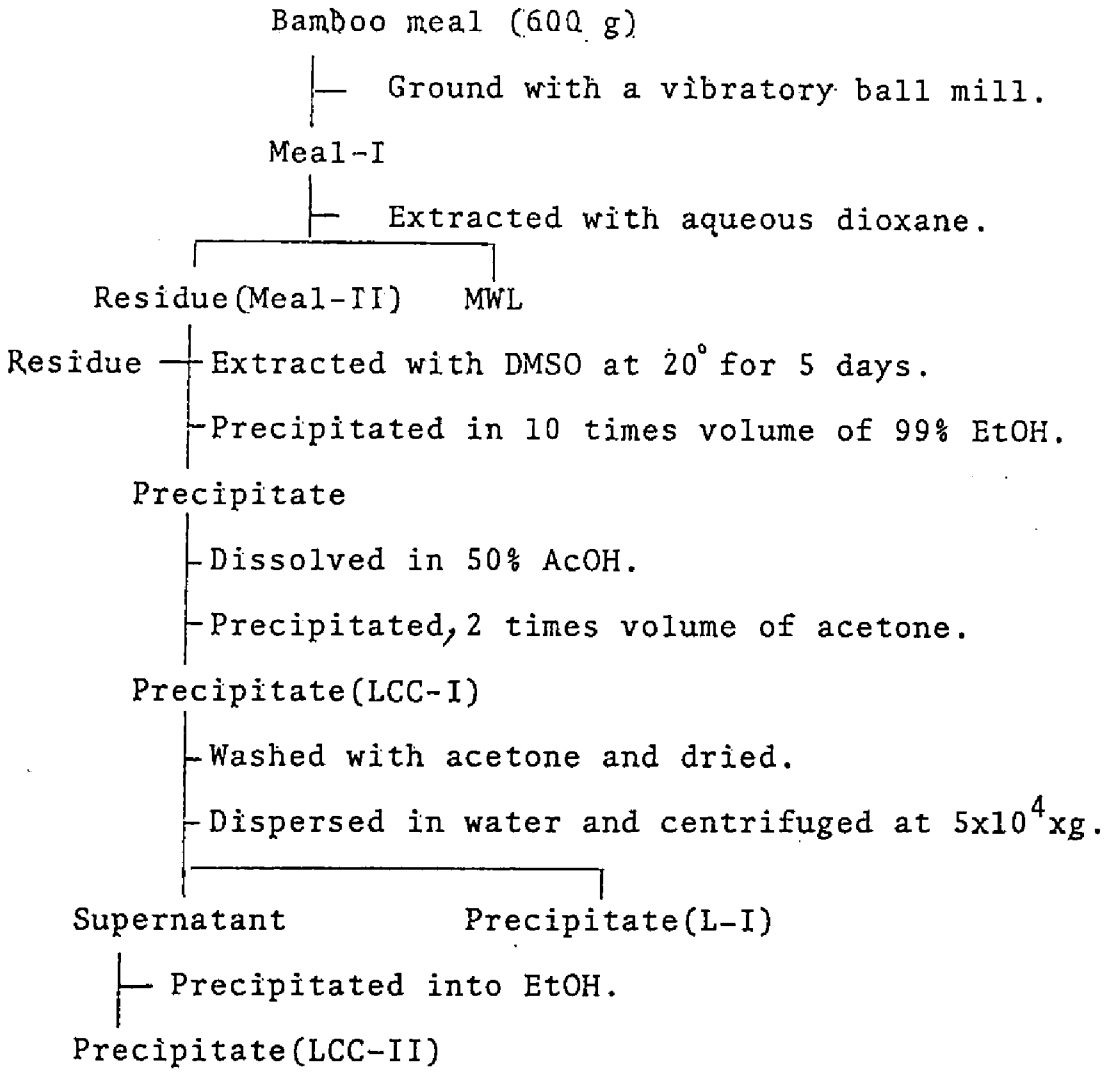
to p-coumaric acid and ferulic acid were cut out and eluted with 95% EtOH and the eluate evaporated to dryness. The concentrate was rechromatographed quantitatively in the same way. Then, p-coumaric and ferulic acids were eluted with 5 ml of 95% EtOH at 50° for 20 min and the absorbance at 310 nm and 323 nm was determined, respectively.

Changes in contents of hydroxycinnamic acid esters and lignin during growth of the shoots:

A young bamboo (Phyllostachys reticulata) 2.5 m in height was used for the experiment. The test samples were taken from five different parts of the shoot. From the three upper parts 50 g each was weighed and 20 g each was weighed from the two lower parts. The tissue samples were homogenized separately with water and the residue obtained by filtration was extracted with EtOH-Bz(1:1). After the extraction five samples were hydrolyzed with 2 N NaOH containing 50 mg of Na-borohydride. The liberated free p-coumaric and ferulic acids were determined as described above. Determination of lignin contents was carried out according to Johnson et al. (52). The results are given in Fig. 13.

Distribution of p-coumarate and ferulate esters within cell wall substances:

Distributions of the hydroxycinnamic acid esters bound to lignin and carbohydrate were examined using the fractions prepared as shown in the following scheme. p-Coumarate and ferulate contents were measured as described above. The lignin contents were determined by the method of Johnson et al. (52).



Scheme 1. PREPARATION OF MWL AND LCC FRACTIONS.

Table 4. DISTRIBUTIONS OF p-COUMARATE AND FERULATE ESTERS WITHIN CELL WALL SUBSTANCES (P. pubescens).

Test samples	p-Coumarate	Ferulate	Lignin*
Meal-I	2.4 %	0.3 %	28.0 %
Meal-II	2.0	0.3	28.4
LCC-I	2.0	0.5	23.8
LCC-II	1.3	0.5	18.2
MWL	7.4	0.1	80.0
L-I	2.9	0.3	40.0

*, Expressed as Klason's lignin content. The values are plotted as shown in Fig.14.

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CHAPTER IV

METABOLIC MECHANISM OF FORMATION OF SYRINGYL COMPONENTS IN LIGNINS

INTRODUCTION

It is presumed that ferulic acid (FA) is a natural intermediate to sinapic acid (SA) since both FA (1-3) and 5-hydroxyferulic acid (5-HFA) (4) were incorporated into syringyl components of lignin molecules. However, following problems still remain to be investigated.

(a) The evidence to demonstrate enzymatic conversion of FA to 5-HFA has not yet been provided.

(b) There might be a misinterpretation on the incorporation of FA-2-^{14}C into syringyl components. Because it is uncertain whether FA administered to plants was incorporated *via* 5-HFA into syringyl components with retention of the methoxyl group or into those *via* caffeic acid (CA) and 3,4,5-trihydroxycinnamic acid (THC) after demethylation and/or demethoxylation of FA as shown in Fig. 1. In fact, the demethylation and the demethoxylation of FA and SA administered to plants were observed by many investigators.

Kratzl and Billek (5) reported that radioactive syringin was incorporated into guaiacyl components of spruce lignin. Reznik and Urban (6,7) recognized the conversion of FA-3-^{14}C

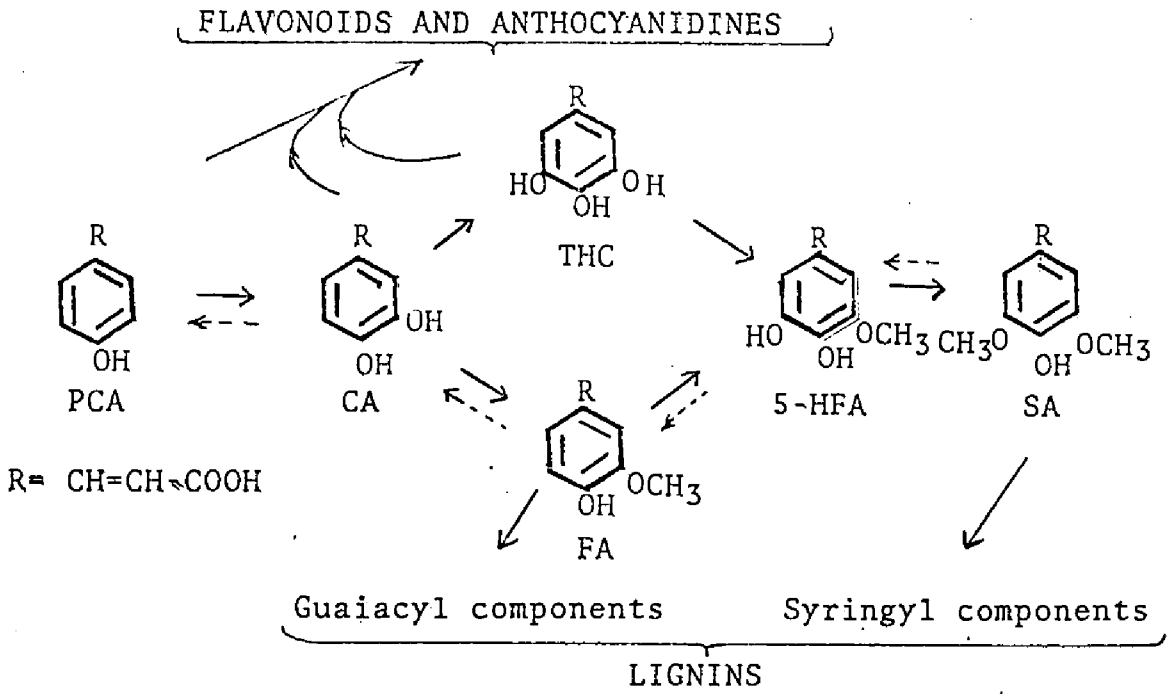

Fig. 1. METABOLIC PATHWAYS OF HYDROXYCINNAMIC ACIDS IN FORMATION OF LIGNINS AND FLAVONOIDS.

to chlorogenic acid in both wheat and red cabbage. Brown and Neish (3) recognized that SA-3-^{14}C was partly incorporated into guaiacyl components isolated as dihdroconiferyl alcohol. Conversion of SA to FA was also recognized by Higuchi and Brown (4). El-Basyouni, Neish and Towers (8) found occurrence of radioactive vanillin and p-hydroxybenzaldehyde after feeding ferulic acid-3-^{14}C and sinapic acid-3-^{14}C to wheat plants. Recently, Steiner has reported an important role of demethylation of SA in biosynthesis of delphinidine in petunia (9). He also observed the conversion of FA to CA and to p-coumaric acid (PCA). These facts show that the methyl ethers of FA and SA, that appear to be chemically stable forms, seem to be easily removed by plants, particularly when such compounds were fed to them for tracer ex-

periments. Therefore, it may be considered that hydroxycinnamic acids are rather interconvertible by modifications of their benzene ring.

(c) THC may serve as a natural precursor for SA . Because THC was found to be a potent substrate to be methylated by bamboo O-methyltransferase (10,11) and Meier and Zenk (12) reported that THC might be formed from CA, showing that THC was more efficiently incorporated into delphinidine than CA.

(d) Then, apart from a metabolical relationship between delhinidine and lignins, it is necessary to clarify metabolic mechanism of formation of SA not only with FA labeled at the side chain or at the benzene ring but also with FA labeled at a methyl group. Because with the former labeled compounds it might be impossible to obtain the information on the removal of the methyl group of FA. However, FA-O-$^{14}CH_3$ was not yet employed in the earlier investigations on lignin biosynthesis except that Hess used this labeled compound for studies on biosynthesis of anthocyanidines in petunia (13).

The present investigation has two aims. The first one is to elucidate whether or not FA administered to bamboo shoot is converted to other hydroxycinnamic acids by the demethylation or the demethoxylation as previously reported (6-9). The second one is to make it clear whether FA-O-$^{14}CH_3$ is incorporated via 5-HFA into syringyl components of lignins with retention of the methoxyl group. At the same time it is elucidated whether or not THC is involved in biosynthesis of lignins as an obligate intermediate.

RESULTS AND DISCUSSION

Radiochromatographic patterns are given in Fig. 2 and 3, showing that FA administered to tissue slices of bamboo shoot was converted to PCA and probably to 5-HFA. However, no radioactive CA was obtained. Radioactive PCA isolated from the chromatogram was crystallized from hot water after addition of 20 mg of the authentic PCA until the specific radioactivity (cpm/mg) was constant (Table 1). Since no changes in the specific activity were observed, it can be recognized that radioactive PCA was converted from FA-2-^{14}C by the demethoxylation. Similar attempts to crystallize radioactive 5-HFA were unsuccessful. The Rf values of hydroxycinnamic acids and location

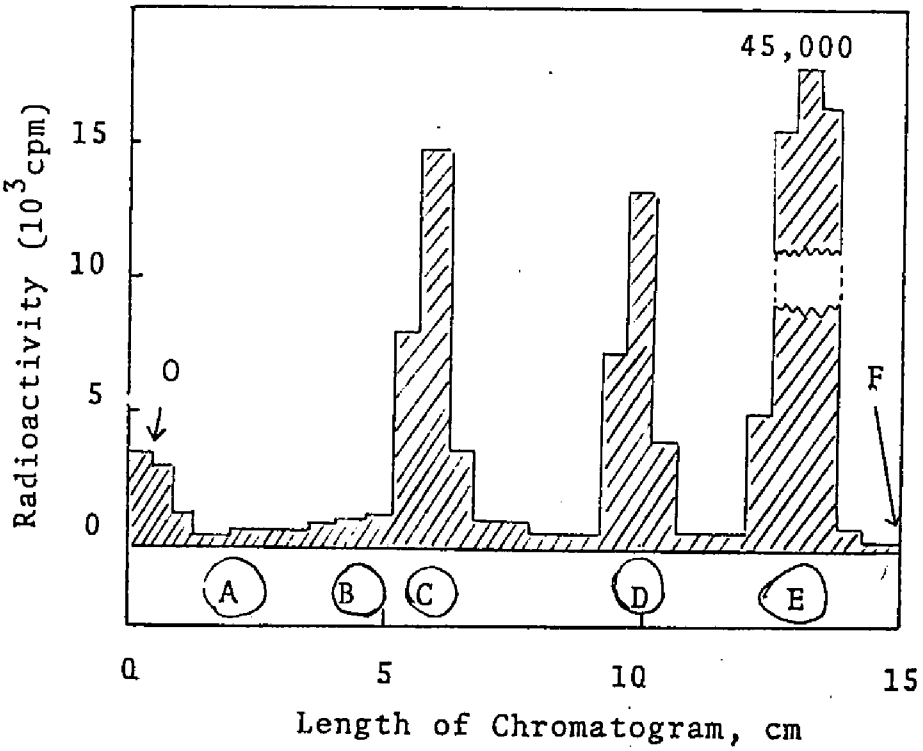

Fig. 2. THIN-LAYER RADIOCHROMATOGRAPHY OF RADIOACTIVE HYDROXYCINNAMIC ACIDS.
Solvent, $CHCl_3$-AcOH-H_2O (2:1:1)
A, THC; B, CA; C,5-HFA; D, p-CA; E, FA

Table 1. CRYSTALLIZATION OF RADIOACTIVE p-COUMARIC ACID FORMED FROM FERULIC ACID-2-^{14}C.

Number of crystallization	Specific activity of PCA (cpm/mg)
1	1,000
2	900
3	1,000
Back ground	60

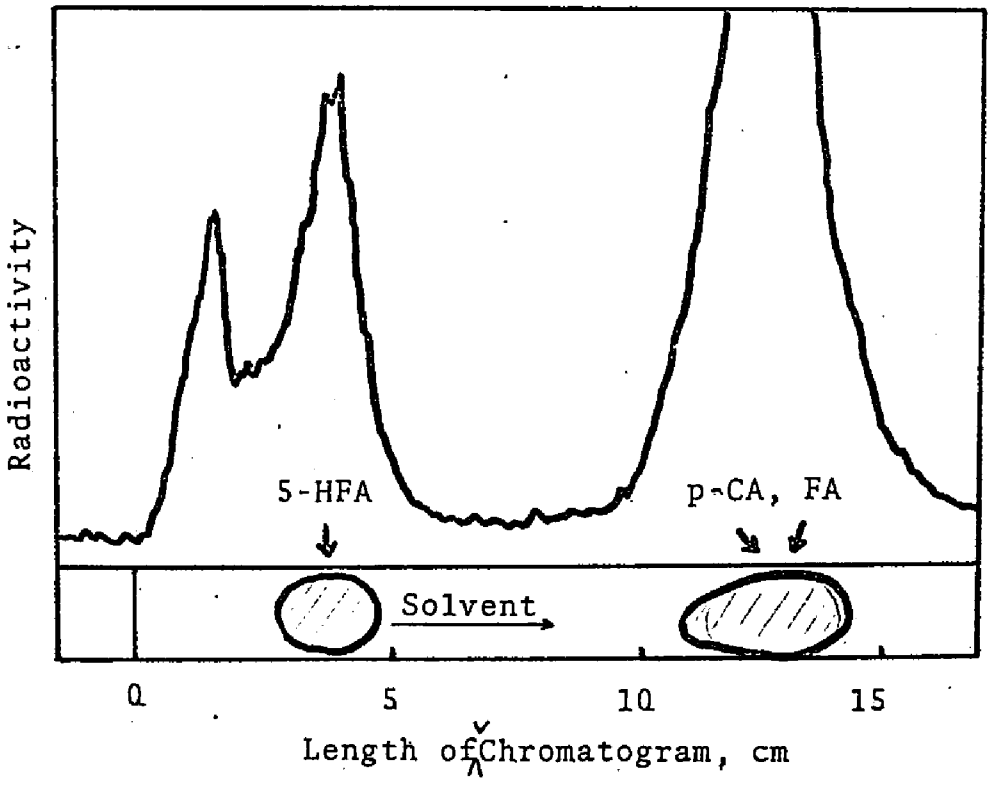

Fig. 3. PAPER RADIOCHROMATOGRAPHY OF RADIOACTIVE HYDROXYCINNAMIC ACIDS.
Solvent, Xylene-methylethylketone-formamide (25:25:1). The paper was used after treatment with ethylacetate-formamide (8:2).

Table 2. Rf VALUES OF HYDROXYCINNAMIC ACIDS AND LOCATION OF THEIR RADIOACTIVITY.

Solvent	PCA	CA	FA	5-HFA	TCA
A	0.60	0.35	0.86	0.50	0.12
B	0.40	0.34	0.54	0.34	0.23
C	0.30	0.07	0.30	0.07	0.02
	Radioactivity located on the chromatograms				
A	+	-	+	+	-
B	+	-	+	+	-
C	+	+	+	+	-

A; TLC, $CHCl_3$-AcOH-H_2O(2:1:1). B; TLC,Toluene-ethylformate-HCOOH(5:4:1). C; PPC, Toluene-AcOH-H_2O(4:1:5).

of their radioactivity are shown in Table 2.

Fig.4. shows the time courses of formation of PCA and compound X (5-HFA?) during incubation of the bamboo tissue with FA-2-^{14}C. Both compounds were found to be formed rather rapidly, showing that about 20% of FA given was converted to them after 100 min incubation. The fact that PCA was converted from FA by the demethoxylation is in good agreement with the results obtained by El-Basyouni et al. (8) and Steiner (9). Although the mechanism of this "demethoxylation" is unknown, it seems that hydroxycinnamic acids (Fig. 1) are relatively interconvertible by resubstitution on their benzene rings. In addition, it must be taken into account in tracer experiments that the demethylation and the demethoxylation may be dependent on physiological conditions of plants to be tested (4).

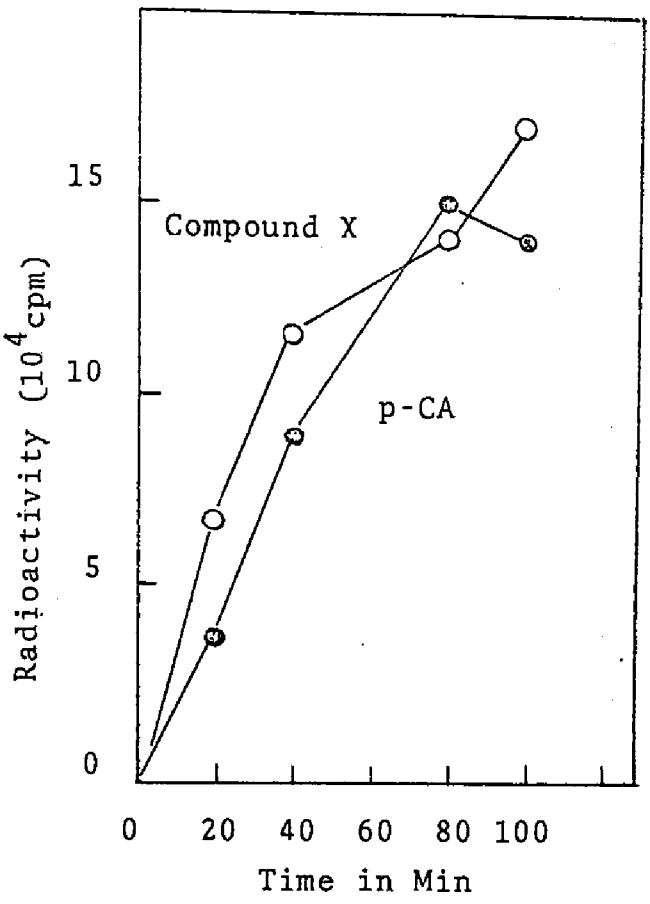

Fig. 4. TIME COURSE OF FORMATION OF p-COUMARIC ACID AND COMPOUND X FORMED FROM FERULIC ACID.

Table 3. CRYSTALLIZATION OF FA-O-$^{14}CH_3$ PREPARED WITH BAMBOO O-METHYLTRANSFERASE.

Number of crystallization	Specific radioactivity of FA-O-$^{14}CH_3$ (cpm/mg)
1	48,000
2	48,900
3	49,000
Back ground	50

Therefore, these phenomena hinted us a methodological importance of using the tracer compound labeled at the methyl group of FA, i.e. FA-O-$^{14}CH_3$, for the biosynthetic studies on lignins. This labeled compound was prepared from CA and S-adenosylmethionine-$^{14}CH_3$ with bamboo O-methyltransferase. The criterion for identification of FA-O-$^{14}CH_3$ was achieved by a crystallization method as shown in Table 3, showing that the constant specific activity was obtained on the third crystallization.

After feeding the various labeled compounds to grass plants, vanillin and syringaldehyde were isolated by nitrobezene oxidation. The yields, specific activity and dilution values for vanillin and syringaldehyde are given in Table 4. The ratio (S/V) for the specific activity obtained with FA-O-$^{14}CH_3$ was found to be 0.33, which was considerably greater than expected. This value is nearly equal to that obtained with phenylalanine-U-^{14}C, indicating that the incorporation rate of FA-O-$^{14}CH_3$ into syringyl components was as great as that of phenylalanine-U-^{14}C. This result also indicates that the demethoxylation did not occur to so a large extent as to affect lowering the specific activity of syringaldehyde derived from FA-O-$^{14}CH_3$. This assumption was further supported by the results obtained from ethanolysis experiment (Table 5). The incorporation rate of FA-O-$^{14}CH_3$ into VMK and SMK was found to be rather greater than any of those of FA-2-^{14}C, CA-2-^{14}C, and Phe-U-^{14}C. And yet no distinct differences in the ratio (SMK/VMK) were observed between them, which is in good harmony with the results obtained on the nitrobenzene oxidation. The ratios (S/V) and (SMK/VMK) obtained in the present investigation are, as a rule, compatible with those

Table 4. INCORPORATION OF FA-O-$^{14}CH_3$ AND PHENYLALANINE -U-^{14}C INTO GRASS LIGNIN.

Compound administered	Yield of aldehydes* (μmole)		Specific activity (μCi/mM)		Dilution	
	V	S	V	S	V	S
FA-O-$^{14}CH_3$(1)	23.3	8.2	0.96	0.32	192	575
FA-O-$^{14}CH_3$(2)	29.3	10.5	1.00	0.33	184	551
Phe-U-^{14}C	17.8	6.6	0.98	0.33	337	1115

*, V; Vanillin. S; Syringaldehyde.

Table 5. INCORPORATION OF THE VARIOUS RADIOACTIVE COMPOUNDS INTO GRASS LIGNIN.

Compound administered	Yield of ethanolysis products (μmole)*		Specific activity (μCi/mM)		Dilution	
	VMK	SMK	VMK	SMK	VMK	SMK
FA-O-$^{14}CH_3$	13.6	7.6	1.52	1.16	121	159
FA-2-^{14}C	12.2	8.3	0.61	0.41	320	481
CA-2-^{14}C**	2.1	1.1	0.99	0.98	270	273
Phe-U-^{14}C**	2.5	1.7	0.97	0.58	340	572

*, VMK; Vanilloyl methyl ketone. SMK; Syringoyl methyl ketone.
**, Most of VMK and SMK were lost by an unexpected accident. However, the values for the specific activity and dilution are valid for comparison with those obtained in other two sets of experiments.

(0.2-1.0) calculated from the values reported in the earlier investigations (1,2,4,14). Then, it can be concluded that most of FA administered to plants was incorporated into syringyl components of lignins without removal of the methyl group, although demethoxylation might occur to some extent. Accordingly, the present result reconfirmed the universal validity of FA as an obligate precursor of SA in biosynthesis of lignins, although Hess already recognized that FA-O-$^{14}CH_3$ was incorporated into SA in formation of anthocyanidine pigments in petunia (13). It may also be concluded that THC is not involved in biosynthesis of lignins in spite of the methylation of THC to SA by bamboo O-methyltransferase. Therefore, it is indicated that hydroxylation must have been brought about at the stage of FA, which is regarded as a diverging step on biosynthetic pathways to lignins of angiosperms and gymnosperms.

MATERIALS AND METHODS

Plant materials:

Sliced tissues from bamboo shoots (Phyllostachys pubescens) 1.5-2.0 m in height were used for the experiment on the demethoxylation of FA-2-^{14}C. Grass plants (Miscanthus sinensis) were used for the experiment on incorporation of various radioactive compounds into lignin molecules.

Radioactive compounds:

L-Phenylalanine-U-^{14}C (405mCi/mM) purchased from Daiichi Kagaku Co., was diluted with cold phenylalanine to 330 μCi/mM. S-Adenosylmethionine(SAME)-$^{14}CH_3$ with the specific activity of 52.3 mCi/mM was procured from New England Nuclear. CA-2-^{14}C

(266 μCi/mM) and FA-2-^{14}C (195 μCi/mM) were synthesized according to the method of Neish (15). FA-O-$^{14}CH_3$ (184 μCi/mM) was enzymatically prepared as described below.

Enzymatic preparation of FA-O-$^{14}CH_3$;

Bamboo O-methyltransferase was obtained according to the procedures previously reported (16). A reaction mixture contained 3.0 ml of the enzyme solution (0.1 M phosphate buffer, pH 8.0) containing 33 mg protein, 0.5 ml of SAME-$^{14}CH_3$(10 μCi), 0.1 ml of cold SAME (0.25 μmole), 0.2 ml of CA (0.5 μmole), 0,1 ml each of 0.1 M $MgCl_2$, 0.1 M NaN_3, and 0.1 M iso-ascorbate. The reaction mixture was incubated for 30 min at 30°. After addition of 10 mg of FA as a carrier and 2.0 ml of 5% HCl into the mixture, FA-O-$^{14}CH_3$ formed was extracted with ether. After evaporation of the ether to dryness, the residue was dissolved in 10 ml of EtOH. 9.5 ml of the EtOH solution was taken and evaporated to dryness, which was used for feeding experiment. The remaining solution (0.5 ml) was analyzed in order to identify FA-O-$^{14}CH_3$ as follows;

(a) The concentration of FA-O-$^{14}CH_3$ in the EtOH was determined by measurement of the absorbance at 323 nm. Alternatively, the radioactivity was measured with a Beckman LS-100 scintillation counter. From both values obtained the specific radioactivity (μCi/mM) of FA-O-$^{14}CH_3$ was calculated.

(b) A portion of FA-O-$^{14}CH_3$solution was submitted to PPC with toluene-AcOH-H_2O (4:1:5) and to TLCs with $CHCl_3$-AcOH-H_2O (2:1:1) and with toluene-ethylformate-HCOOH (5:4:1). After scanning the radioactivity on the chromatograms by use of radiochromatogram scanner (Aloka PCS-4) or the Beckman scintillation counter,

radioactive FA was located on the chromatograms.

(c) To the residual portion of the FA-O-$^{14}CH_3$ solution, 20 mg of cold FA was added and crystallized from hot water until the constant specific radioactivity was obtained.

Experiment on demethoxylation of FA:

The tissue slices (5g) from the bamboo shoots were put into a beaker and 0.5 ml of the solution containing each 10 μmoles of FA-2-^{14}C (1.86 μCi), iso-ascorbate, cysteine, and NaN_3 was infiltrated *in vacuo* into the tissues. The mixture was incubated for 1 hr at room temperature unless otherwise stated. The incubation was run in duplicate. After the incubation the tissues were homogenized with 95% EtOH in a Waring blender and the homogenate was filtered. The filtrate was evaporated and the residue was dissolved in 0.5 ml of 5% $NaHCO_3$, from which an acid fraction containing free hydroxycinnamic acids was obtained by usual extraction procedures. This fraction was submitted to TLC as described above. The radiochromatographs were obtained by measurement of radioactivities in silicagel which was scratched off at 5 mm intervals from the TLC plates.

Experiment on incorporation of the labeled compounds into lignin:

The radioactive compounds such as FA-O-$^{14}CH_3$ (9.0 μCi, 9.5 mg), CA-2-^{14}C (15 μCi, 10 mg), FA-2-^{14}C (9.3 μCi, 10 mg) and phenylalanine-U-^{14}C (20 μCi, 10 mg) in 3.0 ml of water containing 0.2 ml of 5% $NaHCO_3$ were individually administered to four lots of the plants under the sun light and metabolized for 30 hrs after the complete imbibition of the solutions. The former two compounds were fed to 30g of the fresh plants each and the latters to 15g of the fresh plants. The plants in every lot was cut into small

pieces, which were homogenized with hot 95% EtOH. The cell wall residues were used, after extraction with EtOH-bezene (1:1), for alkaline nitrobenzene oxidation and ethanolysis.

Analyses of lignin aldehydes:

The cell wall residues obtained from the plants fed with FA-O-$^{14}CH_3$ and phenylalanine-U-^{14}C was submitted to the alkaline nitrobenzene oxidation by the procedure of Stone et al.(17). The radioactive lignin aldehydes such as vanillin and syringaldehyde were isolated from the reaction mixture by alternate extractions with ether and 20% $NaHSO_3$. The aldehydes were purified by TLCs, which were carried out firstly with water-saturated isopropyl ether, secondly with $CHCl_3$-n-hexane (5:2) and again with the first solvent. The purified vanillin and syringaldehyde were eluted from the chromatograms and determined by the measurement of the absorbances in EtOH at 278 nm and 308 nm, respectively. At the same time, UV-spectra of both aldehydes were taken and identified with those of the authentic specimens. Alternatively, the radioactivities of the aldehydes were measured with the liquid scintillation counter. Their specific activities were consequently calculated from these data.

Analyses of ethanolysis products:

Ethanolysis of the plant residues was carried out according to the procedure by Kratzl et al.(18) except that 20% amount of milled wood lignin (bamboo MWL) on the weight of the rsidue was added to each reaction mixture. Ethanol lignin and crude ethanolysis oil were separated from the reaction mixture as described by these workers. The crude oil was oxidized with ferric chloride in EtOH. The diketones in the resulting mixture were separated

by TLCs, firstly with water-saturated iso-propyl ether and secondly with bezene. Vanilloyl methyl ketone and syringoyl methyl ketone in EtOH were determined by measurement of the absorbances at 326 nm and 320 nm, respectively. The UV-spectra of the two diketones were taken at the same time and identified with those of the authentic specimens. The radioactivities of the diketones were determined with the scintillation counter. The specific activities were calculated from the values obtained.

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CHAPTER V

MECHANISM OF ENZYMATIC FORMATION OF METHOXYL GROUPS IN LIGNIN MOLECULES IN DIFFERENT PLANTS

INTRODUCTION

The incorporation of the methyl group of methionine into the methoxyl groups of lignin was first demonstrated by Byerrum et al. (1) using barley plants. Now that S-adenosylmethionine:catechol O-methyltransferase (E.C.2.1.1.6) from higher plants has been investigated by several workers (2-9), it is established that these O-methyltransferases (OMT) catalyze in the presence of S-adenosylmethionine the methylations of 3,4-dihydroxycinnamic acid (caffeic acid, CA) to 3-methoxy-4-hydroxycinnamic acid (ferulic acid, FA)(2-9), 3-methoxy-4,5-dihydroxcinnamic acid (5-hydroxyferulic acid, 5-HFA) to 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid, SA) (6,7) and 3,4,5-trihydroxycinnamic acid (THC) to both 5-HFA and SA (7-9). These enzyme works support the results obtained with the tracer techniques (10) that such methoxylated cinnamic acids served as lignin precursors. Accordingly, it can be considered that the methoxylations occur at the stage of cinnamate monomers prior to polymerization of coniferyl and sinapyl alcohols.

In connection with the biosynthetic pathways leading to lignins, interest has also been focused on the fact that the lignins of angiosperms consist of both guaiacyl and syringyl units whereas those of gymnosperms hardly contain syringyl units but guaiacyl

units with small amounts of p-hydroxyphenyl units (11-13). It is also interesting to learn that cultured tissues of angiosperms scarecely give rise to syringaldehyde on nitrobenzene oxidation (14-17).

From a biochemical point of view, it is likely that gymnosperms and the cultured tissues lack some enzyme systems required for the formation of syringyl units. Higuchi and Brown (18) showed that labelled FA and 5-HFA were efficiently incorporated into syringyl units of wheat lignin, indicating that the hydroxylation of FA occurs at the 5-position and the subsequent methylation of 5-HFA to SA. Hess (19) demonstrated the transformation of FA-O$^{14}CH_3$ into SA-$^{14}OCH_3$ in seedlings of red cabbage. The author and his associates (20) recognized that FA-O$^{14}CH_3$ was efficiently incorporated into syringyl units of grass lignin with retention of the methyl group. Therefore, this hydroxylation process can be considered as a diverging step which differentiates angiosperm lignins from gymnosperm lignins. However, no evidence for the occurrence of either 5-HFA or "FA 5-hydroxylase" has yet been provided. Apart from this, O-methyltransferase (OMT) may be regarded as one of the key enzymes that are involved in the formation of the methoxyl groups of various plant lignins. Because bamboo and poplar OMTs were found to utilize both CA and 5-HFA as substrates, whereas tissue slices from the shoots of a ginkgo tree selectively methylated CA in the reaction mixture of CA and 5-HFA (8,9). However, OMT has not yet been isolated from gymnosperms and cultured tissues.

The present paper describes the first cell-free extraction of OMT from seedlings of Pinus Thunbergii, the shoots of

Ginkgo biloba and the callus tissues of Salix caprea and Morus bombycis (mulberry). The substrate specificities of these OMTs are discussed in relation to biochemical differences in the methoxyl patterns between angiosperm and gymnosperm lignins.

RESULTS AND DISCUSSION

1. O-Methyltransferases extracted from gymnosperms:

The enzymatic formation of FA,SA and 5-HFA were demonstrated with the cell-free extract from pine seedlings as shown in Figs. 1-4. Similar radiochromatographic patterns were also obtained with the buffer extract from ginkgo shoots. This is the first evidence for the occurrence of OMT in gymnospermous plants. Many attempts to isolate OMT from ginkgo shoots were failed unless bovine serum albumine (2%) was added prior to homogenization of plant tissues. The addition of serum albumine was found to be indispensable for the prevention of the enzyme inactivation. Another demonstration for the enzymatic formation of FA is given in Fig. 5, showing that no FA was formed in the absence of either CA or the extract of Ginkgo biloba. The amount of FA formed increased almost linearly within 60 min of incubation. Alternatively, Fig. 6 shows that the reaction velocity was dependent on the amount of the enzyme solution used. With a doubled amount of the enzyme solution, FA was produced in a doubled amount after 15 min of the incubation. The reaction rate was a little reduced with the incubation time elapsed. This may be due to decrease of the substrate concentration (CA or S-adenosylmethionine) in the reaction mixture. The amounts of the products formed from the various hydroxycinnamic acids with the

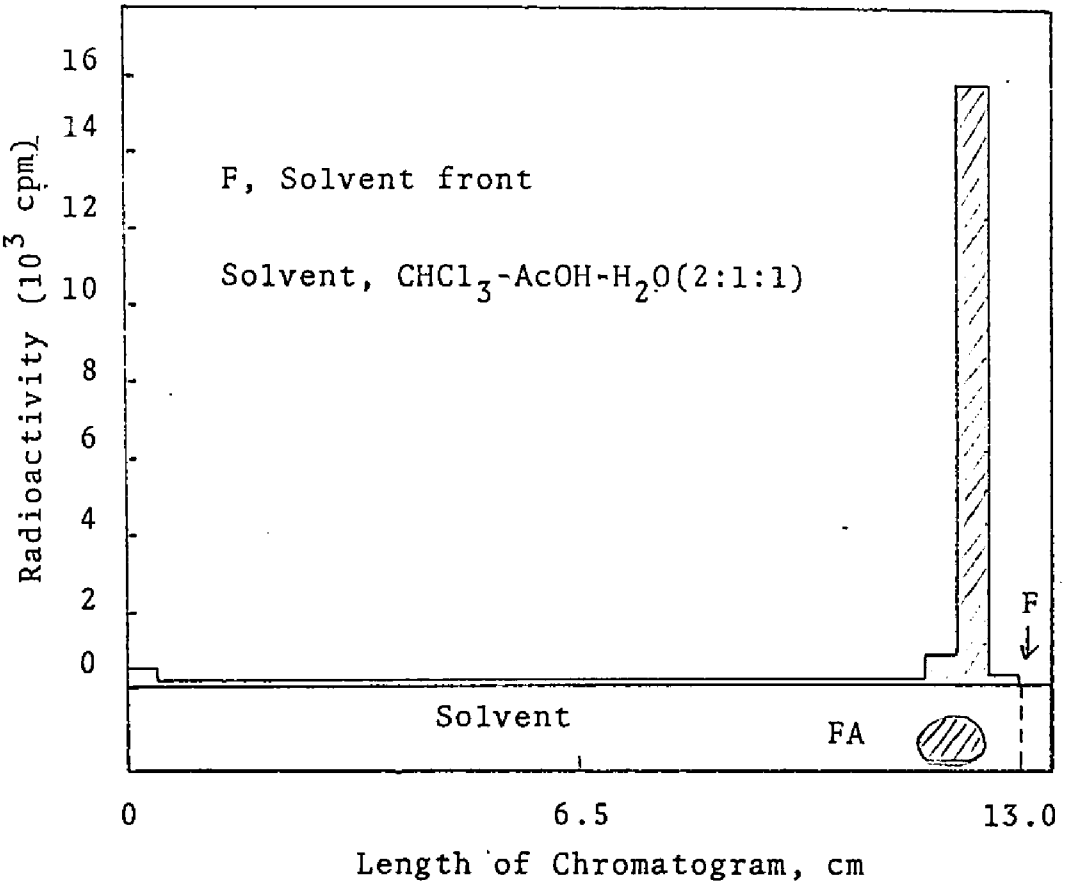

Fig. 1. THIN-LAYER RADIOCHROMATOGRAPHY OF ENZYMATICALLY FORMED FERULIC ACID.

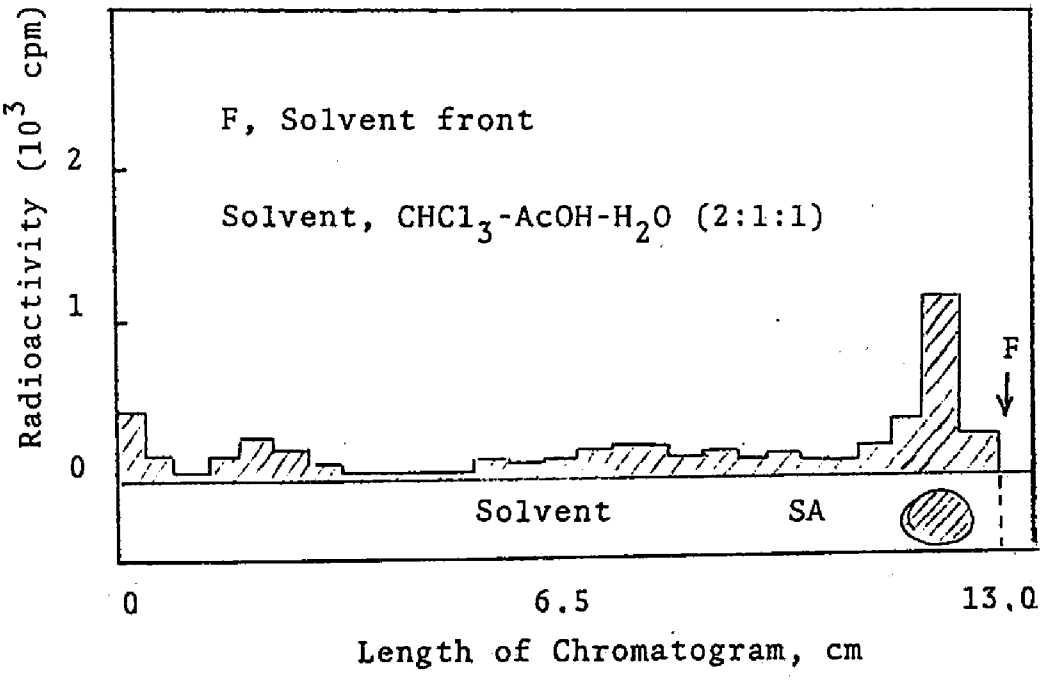

Fig. 2.THIN-LAYER RADIOCHROMATOGRAPHY OF ENZYMATICALLY FORMED SINAPIC ACID.

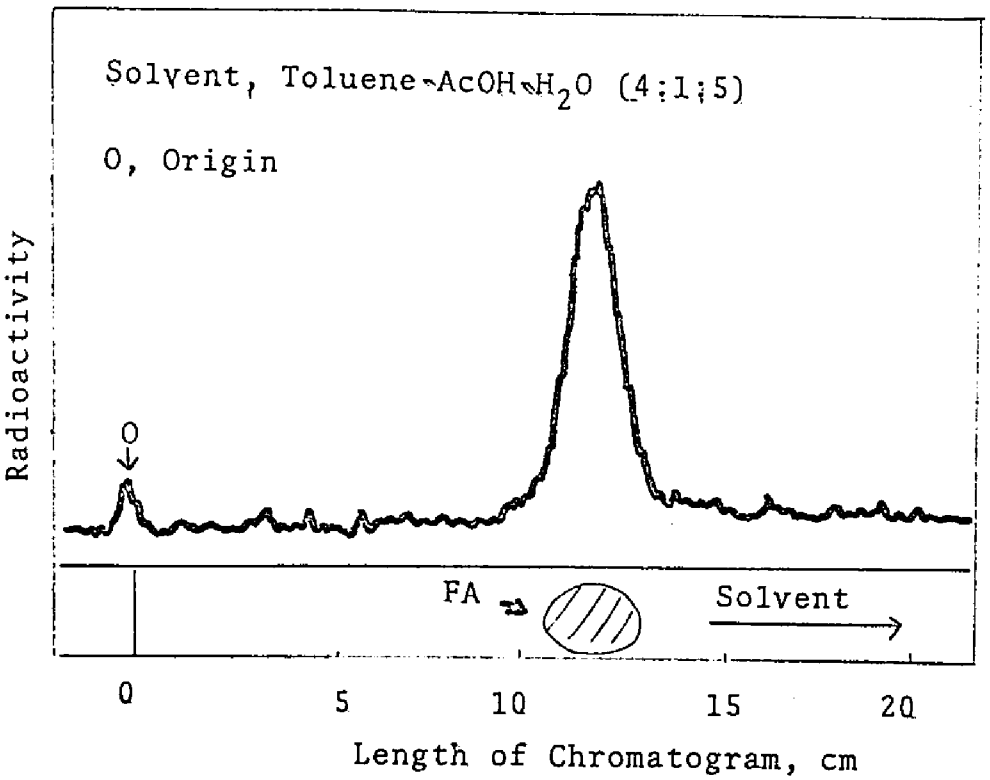

Fig. 3. PAPER RADIOCHROMATOGRAPHY OF ENZYMATICALLY FORMED FERULIC ACID.

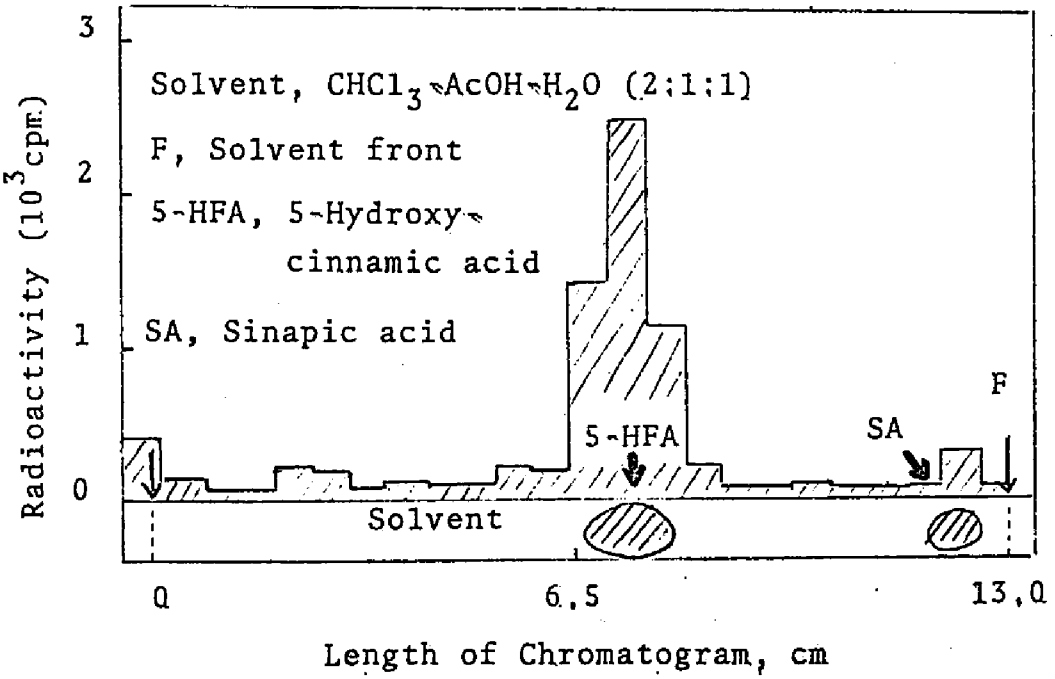

Fig. 4. THIN-LAYER RADIOCHROMATOGRAPHY OF ENZYMATICALLY FORMED 5-HFA AND SA.

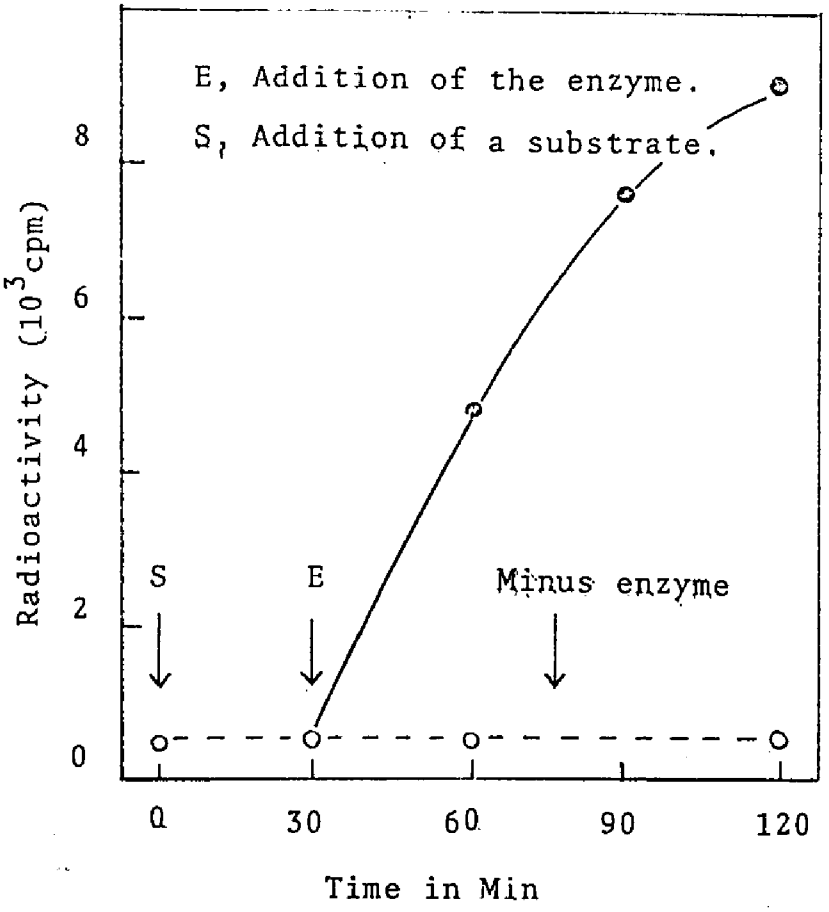

Fig.5. TIME COURSE OF ENZYMATIC FORMATION OF FERULIC ACID (Ginkgo biloba).

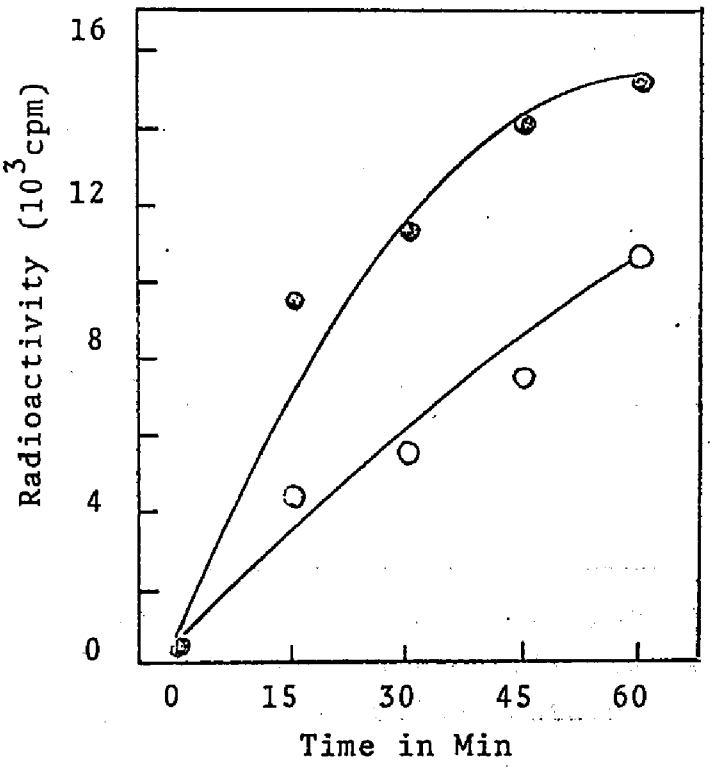

Fig.6. TIME COURSE OF ENZYMATIC FORMATION OF FERULIC ACID FORMATION (Pinus Thunbergii).
-○-, One ml of the enzyme (3.3 mg protein).
-◉-, Two ml of the enzyme (6.6 mg protein).

Table 1. ENZYMATIC O-METHYLATION OF HYDROXYCINNAMIC ACIDS WITH CELL-FREE EXTRACT FROM PINE SEEDLINGS.

Substrates	Products	Radioactivity	n Mole	%
CA	FA	28,435 cpm	74.5	100
5-HFA	SA	3,740 "	8.5	12
THC	5-HFA + SA	10,275 "	26.1	35
Blank, -Enzyme		450 "	0	0
" , -Substrate		600 "	0	0

The reaction mixture was incubated for 90 min at 30°. The enzyme solution used contained 6.6 mg protein. It was confirmed that similar results were obtained by repeated experiments with freshly prepared enzyme solution.

Table 2. ENZYMATIC O-METHYLATION OF HYDROXYCINNAMIC ACIDS WITH CELL-FREE EXTRACT FROM SHOOTS OF A GINKGO TREE.

Substrates	Products	Radioactivity	n Mole	%
CA	FA	17,380 cpm	45.0	100
CA	FA	430 "	0*	0
5-HFA	SA	1,575 "	2.6	6
THC	5-HFA + SA	5,105 "	12.5	28
Blank, -Enzyme		450 "	0	0
" , -Substrate		500 "	0	0

*, This result was obtained with the enzyme preparation without the serun albumine. The reaction mixture was incubated for 90 min at 30°. The enzyme solution contained 2.0 mg protein.

enzyme solution extracted from the pine seedlings and the ginkgo shoots are given in Tables 1 and 2, respectively. It can be seen that both pine and ginkgo OMTs produced FA in the highest yields. On the other hand, SA was formed in the smallest amounts, or in about 10% yield based on FA formed. THC was found to be a more efficient substrate than 5-HFA for the enzymatic methylation. Because the values obtained with THC can be regarded as the yield of 5-HFA on the ground that THC converted largely to 5-HFA accompanied by SA in a very small amount (Fig. 4). Therefore, the effectiveness of the substrate for gymnosperm OMT was lessened in the order of CA, THC and 5-HFA. The same relationship can be observed from the data shown in Figs. 1,2 and 4. Consequently, the fact that gymnosperm OMTs utilize CA preferentially among the three substrates, yielding FA as a guaiacyl component, is very compatible with the fact that gymnosperm lignins consist of mostly guaiacyl units.

2. Substrate specificities of OMTs from various plants including cultured callus tissues:

Fig.7 shows the radiochromatographic patterns of FA and SA which were formed after incubation of CA and 5-HFA with the tissue slices from bamboo and ginkgo shoots in the presence of methionine-$^{14}CH_3$ (9). Bamboo tissue are capable of producing both radioactive FA and SA in a nearly equal amount. On the other hand, ginkgo shoot methylated CA in a much greater amount than 5-HFA, which is in good agreement with the resuls provided with enzyme technique (Tables 1 and 2). Thus, the radiochromatographic data in Fig.7 show that bamboo OMT obviously differs, in behaviours to substrates, i.e. in substrate specificity, from those of pine

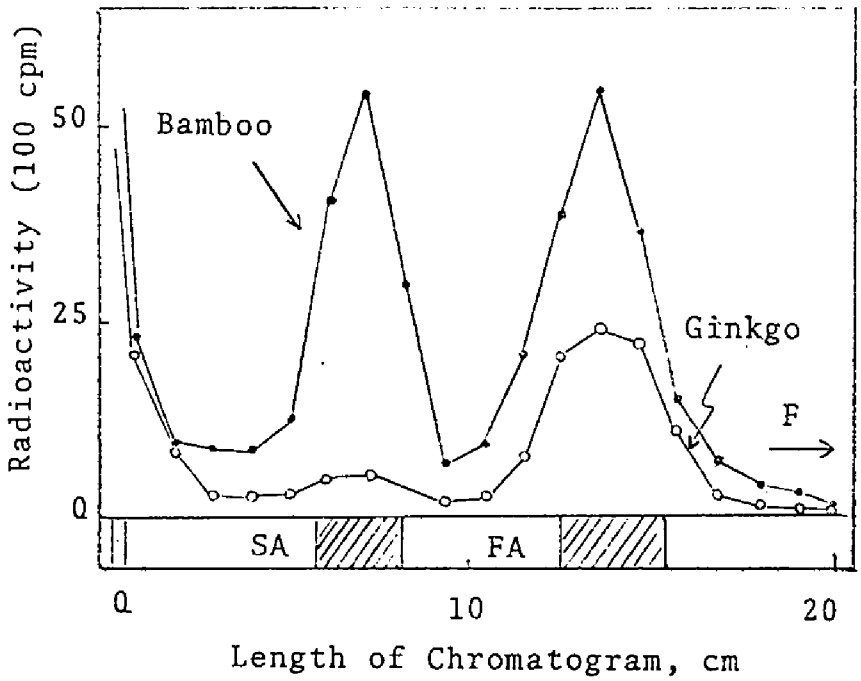

Fig. 7. RADIOCHROMATOGRAPHIC PATTERNS OF FA AND SA FORMED AFTER INCUBATION OF CA AND 5-HFA WITH THE TISSUE SLICES FROM BAMBOO AND GINKGO SHOOTS IN THE PRESENCE OF METHIONINE-$^{14}CH_3$.
F , Direction of the solvent, toluene-AcOH-H_2O(4:1:5).

and ginkgo.

Another radiochromatographic patterns which were obtained with the cell-free extract from the cultured tissues of Salix caprea are shown in Fig.8. It can be suggested from the sizes of the two peak areas that SA was formed in a nearly double amount as compared with FA. This suggestion was turned out to be true by the data given in Table 3, showing the formation of FA and SA during different incubation times by OMTs from the callus tissues of Salix caprea and Morus bombycis. It can be seen from the Table that the amounts of SA was twice as great as that of FA. For the comparison with the data in Table 3, the

amounts of FA and SA formed by bamboo OMT during different periods of incubation are given in Table 4.

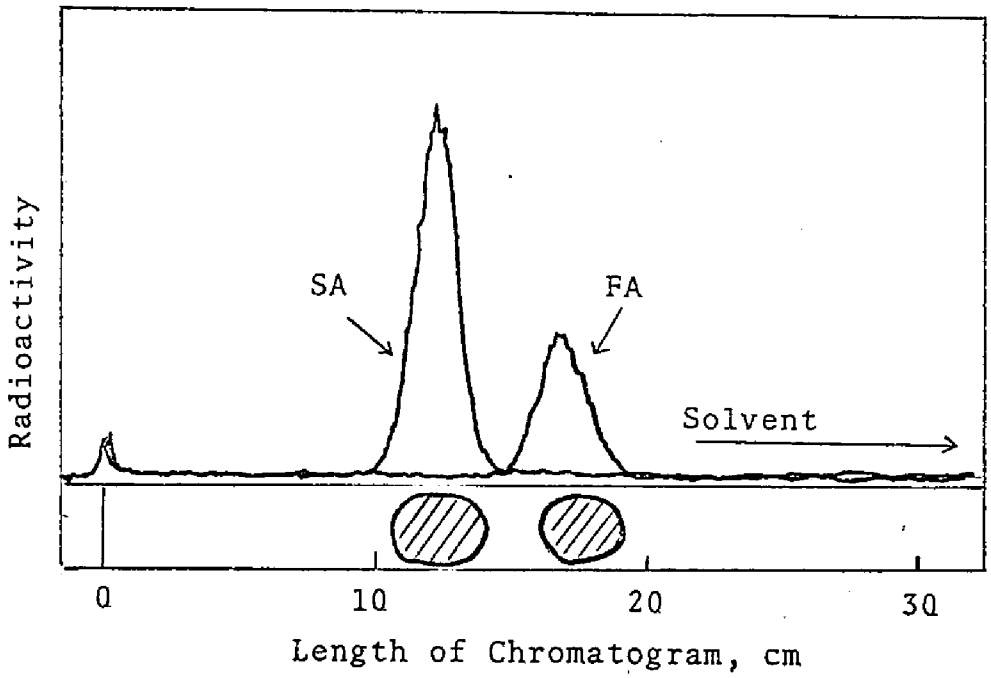

Fig. 8. PAPER RADIOCHROMATOGRAPHY OF ENZYMATICALLY FORMED SA AND FA (*Salix caprea*).
Solvent, toluene-AcOH-H_2O(4:1:5,organic layer)

Table 3. ENZYMATIC FORMATION OF FA AND SA DURING DIFFERENT PERIODS OF INCUBATION.

	Incubation time (min)	Products (n Mole) FA	SA	Ratio (SA/FA)
Salix caprea	0	0	0	-
	30	20	35	1.8
	60	35	53	1.5
	90	40	64	1.6
Morus bombycis				
	30	17	27	1.6
	60	27	50	1.9
	90	35	61	1.7

The assay conditions were the same as described in the text except that the incubation time was altered as shown.

Table 4. ENZYMATIC FORMATION OF FA AND SA DURING DIFFERENT PERIODS OF INCUBATION (Bamboo).

Incubation time (min)	Products (n Mole) FA	SA	Ratio (SA/FA)
0	0	0	-
5	64	75	1.2
10	95	124	1.3
15	133	130	1.0
20	153	186	1.2
30	195	213	1.1
60	213	208	1.0

Bamboo OMT was prepared as described in the previous paper(9). The enzyme solution (2 ml) used contained 20 mg protein.

Table 5. THE RELATIONSHIP BETWEEN THE SUBSTRATE SPECIFICITIES OF VARIOUS PLANT O-METHYLTRANSFERASES AND THE RATIO OF LIGNIN ALDEHYDES (S/V).

Source of OMT	Products(n Mole)** FA	SA	Ratio (SA/FA)	Lignin aldehydes (S/V)
Bamboo	100	100-120	1.0-1.2	1.1
Poplar*	100	300	3.0	1.5
Pine	100	10	0.1	≈0
Ginkgo	100	10	0.1	≈0
Callus tissue of,				
S. caprea	100	170	1.7	0.1
M. bombycis	100	180	1.8	1.0

*, The extraction and the assay conditions were described in the previous paper(8).

**, The amounts of the products formed are expressed as the relative values calculated from the data previously obtained.

The relative values of FA and SA formed by various plant OMTs and the ratio of syringaldehyde to vanillin (S/V) that are obtained on nitrobenzene oxidation of the plants used for the source of OMT are shown in Table 5. Bamboo and poplar OMTs gave 1.0 and 3.0, respectively, for the ratio (SA/FA) while pine and ginkgo gave 0.1. These values appear to be very indicative of the presence of a correlation between the substrate specificities of the above-mentioned OMTs and contents of guaiacyl and syringyl units in angiosperm and gymnosperm lignins. Particularly, the ratio obtained with ginkgo and pine are quite compatible with the fact that gymnosperm lignins contain at best about 10% syringyl units. On the other hand, the callus tissues from Salix caprea and mulberry gave greater values of the ratio (SA/FA) inspite of their lower ratios (S/V), 0.1 for Salix caprea in particular. The ratio (S/V) for mulberry was considerably greater than that for other callus tissues (15). This discrepancy may be due to other unknown factors because the callus tissues were grown under the physiologically different conditions as compared with natural vascular plants capable of forming xylem tissues.

In conclusion, one of the reasons for the different methylations of the hydroxycinnamic acids can be explained in terms of differences of the substrate specificities of angiosperm and gymnosperm OMTs. Therefore, OMT involved in biosynthesis of various plant lignins is considered as one of the key enzymes, which regulate the formation of the guaiacyl and syringyl lignins in higher plants.

MATERIALS AND METHODS

Plant materials:

Pine seedlings (Pinus Thunbergii) were utilized for the extraction of OMT. The seeds were germinated on wet vemiculite under the light of 2×10^4 lux for 30 days.

Young shoots of Ginkgo biloba were sampled in the early summer at the campus of Kyoto University.

The callus tissues of Salix caprea and mulberry (Morus bombycis) were cultured in the media of Heller, and Skoog and Murashige (21).

Extraction of OMT:

Plant materials (10g of the seedlings or ginkgo shoots, 30g for the callus tissues) were homogenized carefully in the ice-cooled mortar with 10 ml of 0.1 M phosphate buffer (pH 8.0) containing 0.1 M NaN_3, 0.1 M cysteine, 0.1 M $MgCl_2$ (0.5 ml each), and bovine serum albumine (200 mg). Polyclar AT (1g) was added on the homogenization. The homogenate was filtered and centrifuged at 14,000 rpm for 30 min at 0°. The supernatant solution (10-20 ml) obtained was used as an enzyme preparation for the assay of OMT activity. Enzyme protein concentration was determined spectrophotometrically on the buffer extract without the serum albumine added.

The extraction of OMT from bamboo (Mohso) (9) and poplar (8) were described in the previous papers.

Assay of OMT activity:

The reaction mixture contained 2 ml of the enzyme solution, 0.1 ml of a substrate (0.5 μmole of CA, 5-HFA, or THC) and 0.1 ml of S-adenosylmethionine-$^{14}CH_3$ (0.25 μmole, 0.05 μCi). The reaction

mixture was incubated for 90 min at 30° unless otherwise stated. Radioactive products were extracted with ether (3x5 ml) after addition of 0.5-1.0 ml of 5% HCl and cold FA or SA as a carrier into the mixtures. After evaporation of the ether, the residue was directly used for the measurement of the radioactivity without carrying out TLC or PPC. Each residue was dissolved in 1.0 ml of dioxane and transferred in a vial tube containing toluene scintillator solution (6 ml). Then, the radioactivities of the products were determined with a Beckman scintillation counter (LS-100). The activity of OMT was expressed as nano mole of FA or SA formed by 2 ml of the enzyme solution. The amounts of the products were calculated from the radioactivities measured; 10^4 cpm is equivalent to 26.6 n moles of a product.

Identification of the radioactive products:

The ether extract from the reaction mixture was submitted to PPC with toluene-AcOH-H_2O (4:1:5) or to TLC with $CHCl_3$-AcOH-H_2O (2:1:1). From the Rf values of the products and from the radiochromatographical data shown in the Figures in the text, the products were identified with authentic FA, SA, or 5-HFA, respectively.

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CHAPTER VI

ESTER LINKAGES OF p-COUMARIC ACID IN BAMBOO AND GRASS LIGNINS

INTRODUCTION

It has been known as a unique feature of bamboo and grass lignins that they contain 5-10% of esters of p-coumaric acid accompanied by ferulic acid in small amounts (1). Kuc and Nelson (2) investigated the variation of p-coumaric and ferulic acid contents in lignin during growth of maize as part of a genetic and biochemical study of maize hybrids. It is of interest to elucidate the structural pattern of the ester linkages of p-coumaric acid in graminae plant lignins with respect to their biochemical formation.

The occurrence of the esters of p-coumaric acid in sugar cane and of p-hydroxybenzoic acid in aspen lignins was first demonstrated by Smith (3,4), indicating that p-hydroxybenzoic acid is linked to aliphatic hydroxyl groups.

As for the structural pattern of the ester linkages of p-hydroxybenzoic acid in aspen lignin, Nakano et al. presumed that part of the acid is esterified with a hydroxyl in the α-position (benzyl carbon) of the side chain of lignin molecules (5, 6). Okabe and Kratzl also proposed the possibility that p-hydroxybenzoic acid might be linked to the α-position. Their supposition is based on the mechanism of the reaction where the conjugate base of the acid attacks nucleophilically the α-carbon atoms of "intermediary quinone methides" (7), and they concluded that some of the ester linkages of p-hydroxybenzoic acid in aspen lignin are formed according to this reaction mechanism. Sarkanen et al.

have recently found acetyl groups in lignins of various gymnosperms and angiosperms (8,9), indicating that such acetyl groups are formed as suggested by Kratzl et al.

Pew et al. proposed another possibility of ester formation on the grounds that a new type of ester compound (10) was isolated from dehydrogenation products of propiophenone after a peroxidase-catalyzed reaction.

Since the esters of p-coumaric acid (PCA) and p-hydroxybenzoic acid fcund in bamboo and aspen lignins, respectively, do not occur in other species of woody plants, the present author and his associates consider that the formation of these esters is a biological feature depending on plant species and that it is biochemically controlled by the plants. In the present paper a different type of the ester linkage of PCA in bamboo and grass lignins is proposed on the basis of analytical data obtained with model substances and natural lignins.

RESULTS AND DISCUSSION

Spectral analysis of ester linkages:

On the basis of the analytical data of hydrolysis experiment and titration curves of aspen lignin, Smith concluded that p-hydroxybenzoic acid molecules might be linked by ester linkages to aliphatic hydroxyl groups in the lignin molecules(4). However, definite evidence for the structural patterns of ester linkages of PCA with bamboo and grass lignins have not yet been provided, although the ester carbonyl was observed in IR spectra and the acid is liberated on alkaline hydrolysis (1-3).

Figure 1 shows characteristic pH-absorbancy curves of PCA,

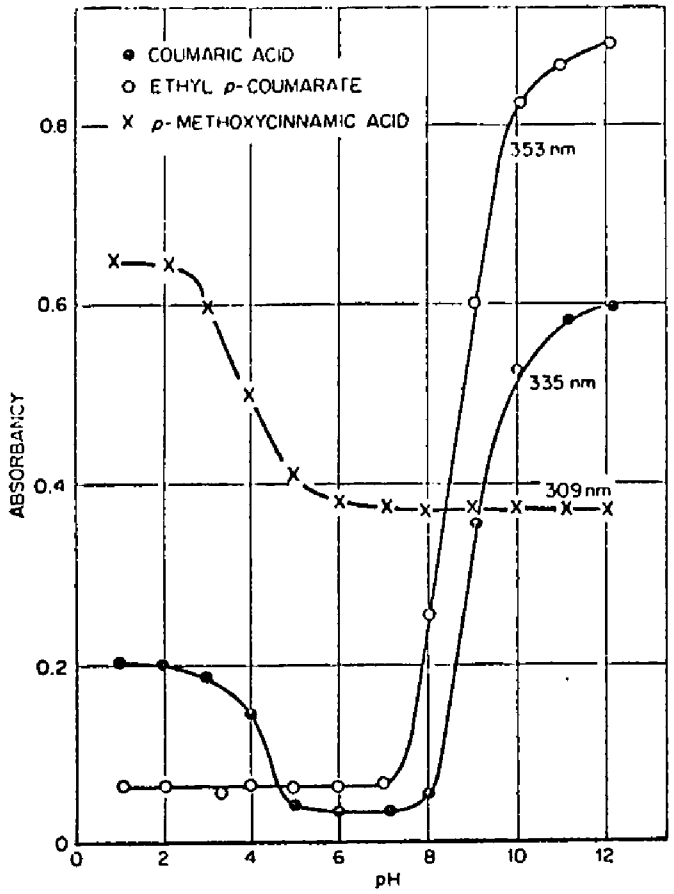


Fig. 1. pH–absorbancy curves for *p*-coumaric acid derivatives.

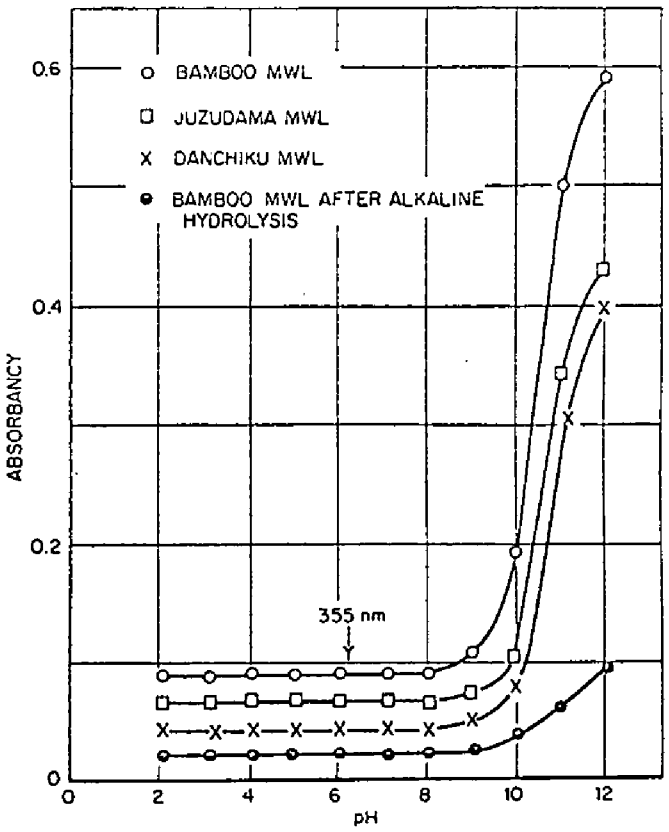


Fig. 2. pH–absorbancy curves for various MWL preparations.

p-methoxycinnamic acid, and etyl ester of PCA. PCA, having two dissociable groups, shows two inflection points around pH 4 and 9. On the other hand, p-methoxycinnamic acid and ethyl ester of PCA, having only one dissociable group, give one inflection point around pH 3 and 9, respectively. By comparison of the absorbancy curves of milled-wood lignin (MWL) shown in Fig.2 with those of model compounds, it is found that the MWL shows similar inflection to that of ethyl p-coumarate in the alkaline pH range. After hydrolysis of the MWL, a significant increase in the absorbance was no longer observed . Therefore, the ester

linkages in bamboo and grass lignins are formed between carboxyl groups of the acid and hydroxyl groups of lignin molecules, which is in accordance with the result obtained by Smith (4).

Effects of organic acids on formation of esters in DHP:

As already mentioned, Sarkanen et al. reported the presence of acetyl groups in both angiosperm and gymnosperm lignins on the basis of detailed analysis of their IR spectra (8,9). It was found that such acetyl esters were formed when coniferyl alcohol was dehydrogenated in acetate buffer solutions of pH 5.5.

As shown in Fig.3 IR spectra of dehydrogenation polymer (DHP) preparations formed when the reaction mixtures contained a given organic acids show a significant absorption band due to ester carbonyl at 1730 cm^{-1}, except for the spectrum of DHP prepared in the presence of cinnamic acid-2-^{14}C. On the other hand, DHP produced in distilled water without any organic acids gives no significant absorption band around 1730 cm^{-1}.

Although these spectra do not directly support that the prediction that the aliphatic ester groups are attached exclusively to the α-carbon atoms, it seems reasonable to consider that these carbonyl absorption bands are due to esters formed as a result of addition of aliphatic acids to the α-carbon atoms of "intermediary quinone methides". In order to examine this question further, the IR spectrum of DHP-B was again taken after methanolysis, by which

Table I. Incorporation of Radioactive Organic Acids into DHP[a]

Radioactive organic acids	*Incorporation, %*[b]
Acetic acid-1-^{14}C	3.6[c]
Acetic acid-1-^{14}C	0.4–1.6
Cinnamic acid-2-^{14}C	0.4
p-Coumaric acid-2-^{14}C	6.0
Ferulic acid-2-^{14}C	1.7

[a] DHP was prepared as described in the text.
[b] The amounts of the organic acids incorporated are expressed in percent based on dry weight of DHP.
[c] This high value was obtained only when DHP was formed in 0.1 *M* acetate buffer solution at pH 5.0.

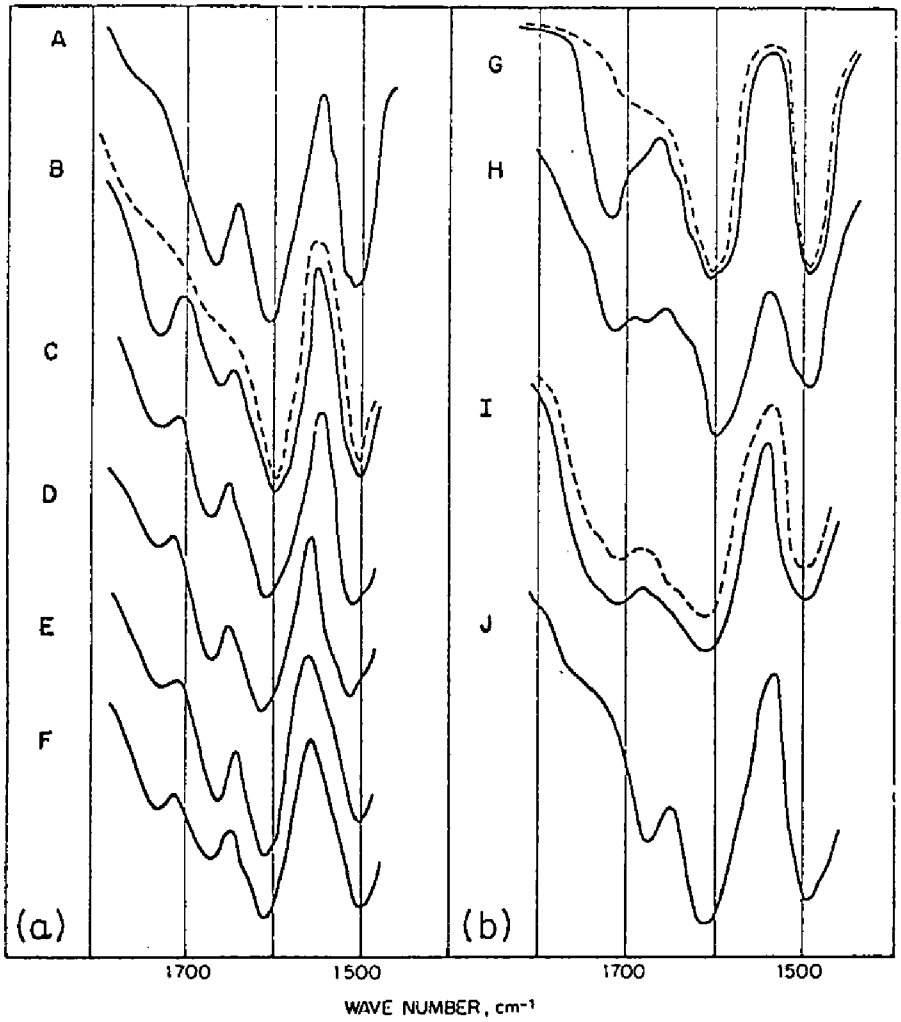


Fig. 3. IR spectra of various DHP preparations and bamboo lignins. Curve A represents the spectrum of the DHP formed in the absence of any organic acids. B, C, D, E, and F are the IR spectra of DHPs prepared in the presence of acetic, propionic, *n*-butyric, acrylic, and succinic acids, respectively. The dotted line in part (a) indicates the spectrum taken after methanolysis of DHP-B. G and H are IR spectra of bamboo MWL and BNL, respectively. I and J represent IR spectra of DHPs prepared in the presence of *p*-coumaric and cinnamic acids, respectively. The dotted lines in part (b) indicate the spectra obtained after alkaline hydrolysis.

ester groups attached to the α-carbon atoms are replaced by methoxyl groups. Since the absorption band around 1730 cm^{-1} was reduced significantly after the methanolysis, as shown by the dotted line in Fig.3 (a), acetyl esters were removed by the methanolysis. Therefore, such esters had been linked to the benzyl α-carbon atoms.

However, as far as the esters of PCA in bamboo and grass lignins are concerned, there seem to exist other structural

patterns for the ester linkages. Infrared spectra of bamboo MWL and Brauns' native lignin (BNL), and DHP prepared in the presence of PCA and cinnamic acids are shown in Fig.3 (b). After alkaline hydrolysis of bamboo MWL and DHP-I, the former lost the absorption band at 1730 cm^{-1}, whereas the latter showed only partial loss. These results indicate that PCA contained in DHP is involved in linkages different from the ester linkages occurring in bamboo MWL and in DHP preparations formed in the presence of aliphatic acids.

Incorporation of labeled acetic and cinnamic acids into DHP:

As already described, it is doubtful whether the ester linkages of PCA are formed at only the α-carbon atoms of "intermediary quinone methides", in the same manner as found in the addition of aliphatic acids. Thus, when coniferyl alcohol is dehydrogenated in the presence of PCA, the hydroxyl group of the acid is also affected by peroxidase, so that free radicals are produced which copolymerize with coexisting radicals of dehydrogenated coniferyl alcohol. It was found that similar copolymerization occurred on preparation of DHP in the presence of p-hydroxybenzoic acid (7). In connection with the IR spectral data shown in Fig.3 contents of ^{14}C-labeled acetic, cinnamic, and ferulic acids and PCA incorporated into DHP were calculated from their radioactivities (Table 1). On the basis of repeated experiments, it was found that when these organic acids were added in equimolar or in half-molar concentration, as compared with that of coniferyl alcohol, the amounts of incorporated acids, except for PCA and ferulic acid, were relatively small. However, a large amount of acetic acid-1-^{14}C,

3.6%, was incorporated when DHP was formed in 200 ml of 0.1 M acetate buffer solution containing 2 mmoles of coniferyl alcohol. Therefore, the amount of aliphatic acid incorporated into DHP seems to be dependent, to some extent, on the concentration of organic acid added to the reaction mixtures. Among the aromatic and aliphatic acids tested, PCA was incorporated in the greatest amount (6%) in spite of the low concentration, which is in accordance with the copolymerization mechanism just indicated.

Figure 4 shows the UV spectra of the radioactive DHP containing 6% of PCA and the natural bamboo MWL. The former can clearly be distinguished from the latter. Therefore, PCA incorporated into DHP is not linked by the same ester linkage as that found in bamboo and grass lignins. However, it is likely that cinnamic acid, in spite of the low degree of incorporation, is linked to the α-position in the ester form described above, because it is not affected by peroxidase. This assumption concerning the linkage formed by cinnamic acid with the α-carbon atoms is examined by the following methanolysis experiment.

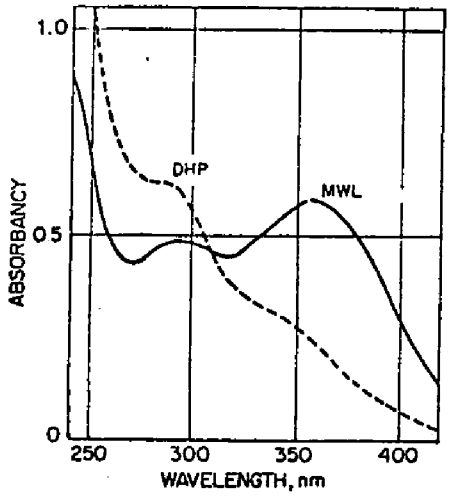


Fig. 4. UV spectra of bamboo MWL and DHP in 0.1*N* NaOH. DHP contained 6% *p*-coumaric acid-2-^{14}C.

Model experiments for methanolysis and alkaline hydrolysis of radioactive DHP:

In order to determine the position (α or γ) of PCA ester on the side chain of the lignin molecules, it is appropriate to use methanolysis with 0.5% methanolic hydrogen chloride and thioglycolic acid treatments (mercaptolysis) since these reagents attack mainly the α-carbon atoms of the side chain of the lignin molecules, giving the benzyl methylether and thioether (18,19), respectively. The former method was used by Freudenberg (20) and Adler (21) to estimate the number of benzylalcohol groups and arylether structures in lignin. Model experiments on methanolysis of guaiacylglycerol-β-guaiacyl ether triacetate (GGT), which contains one acetyl ester at the α-carbon atom, were performed. After the methanolysis the methoxyl content increased to approximately the theoretical value (theor. 21.1%, found 19.2%), providing evidence that the acetyl group at the α-position can be removed by methanolysis.

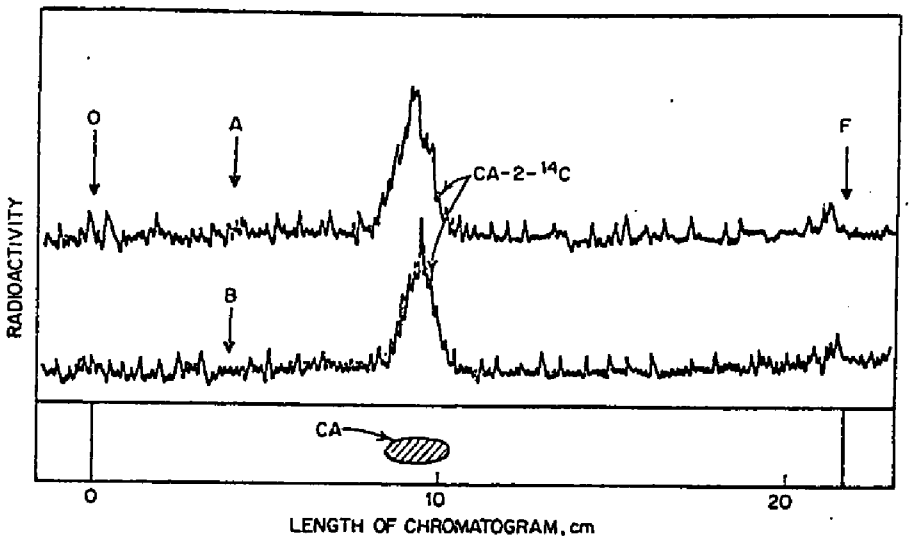


Fig. 5. Radiochromatogram of cinnamic acid-2-^{14}C liberated from radioactive DHP after alkaline hydrolysis and methanolysis. A = radiochromatogram of alkaline hydrolysis product, B = radiochromatogram of methanolysis product, CA = authentic cinnamic acid, O = origin, F = solvent front.

Furthermore, this is in accordance with the fact that the absorption band around 1730 cm^{-1} disappeared after methanolysis of DHP-B, as shown in Fig.3 (a). Therefore, if PCA is located at the α-postion, it will be liberated on methanolysis. On the other hand, it will not be liberated if it is associated with the terminal γ-position. The radioactive DHPs containing cinnamate- and PCA-2-^{14}C can be used as model substances because the incorporated acids should be removed on both alkaline hydrolysis and methanolysis, if they are attached as ester groups at the α-position. The incorporated cinnamic acid was actually released by both treatments, as shown in the radiochromatograms of Fig.5. On the other hand, PCA was not liberated on alkaline hydrolysis (Fig.6) but only on methanolysis (Fig.7). The fact that the DHP containing PCA gave several radioactive fragments as well as free PCA after the methanolysis is in accordance with an indication of copolymerization of PCA during the dehydrogenation of coniferyl alcohol. From these results it may

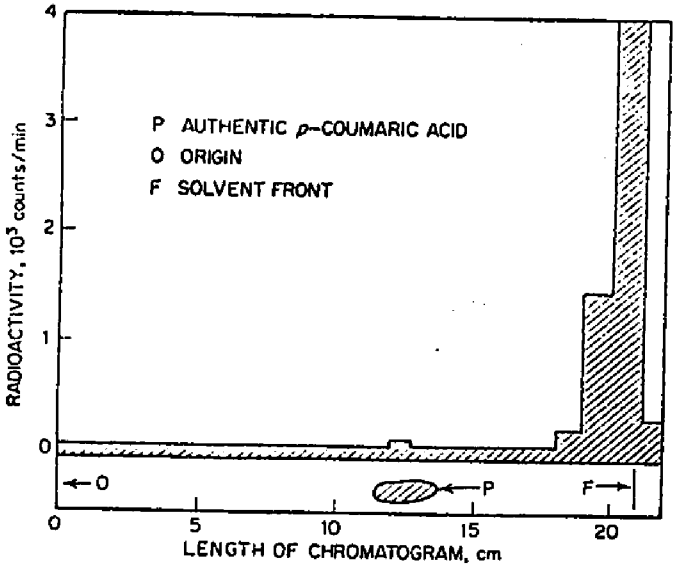


Fig. 6. Radiochromatogram of alkaline hydrolysis products from radioactive DHP.

be concluded that cinnamic acid is linked to the α-position in the ester form, whereas PCA incorporated into DHP is not bound in the ester form.

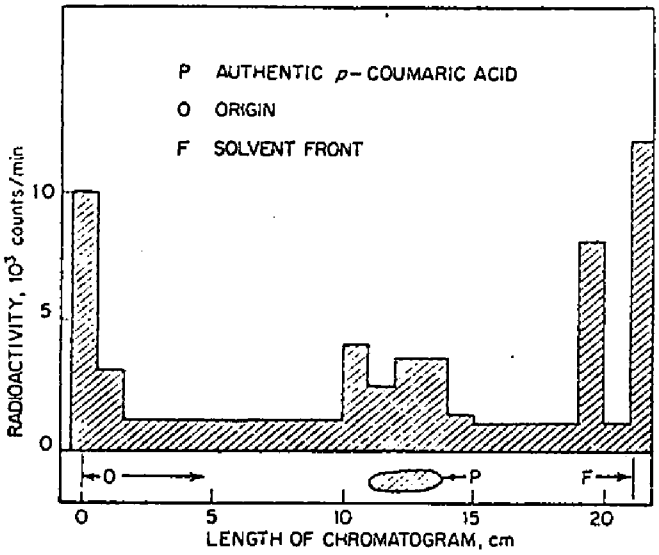


Fig. 7. Radiochromatogram of methanolysis products from radioactive DHP.

UV-Spectra of variously treated MWL preparations:

Figure 8 shows UV spectra of variously treated bamboo MWL preparations. A characteristic peak at 355 nm in the spectra did not change after methanolysis and thioglycolation of the MWL, whereas it disappeared after alkaline hydrolysis. Since the peak did not disappear after refluxing the MWL in 0.5% methanolic hydrogen chloride for 48 hr, the ester linkages of PCA are extremely resistant to such methanolysis. This resistance also suggests the presence of the ester linkages at the γ- rather than the α-carbons.

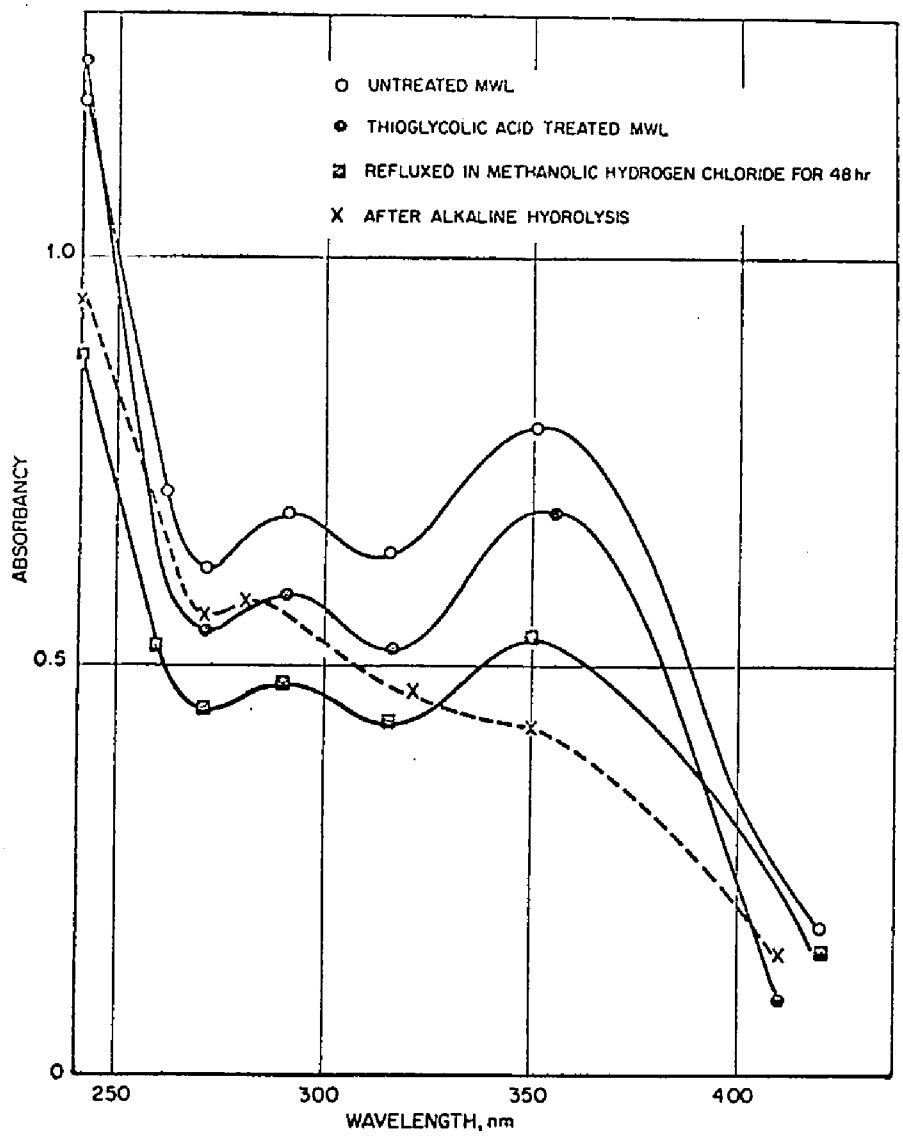


Fig. 8. UV spectra of variously treated bamboo MWL (0.1*N* NaOH).

Methanolysis and thioglycolation of various lignins:

Table II shows the contents of the ester of PCA and methoxyl groups in bamboo MWL before and after methanolysis of the MWL. The contents of methoxyl groups increased from 14.5 to 21.5%, or from 0.94 to 1.40 OCH_3 per phenylpropane unit. After methanolysis at the refluxing temperature, additional methoxyl groups were introduced into the lignin. On the other hand, the content of PCA did not change after methanolysis. These data are consistent with the UV-spectral data shown in Fig.8.

Table III gives the contents of PCA in various lignins

Table II. Content of p-Coumaric Acid Ester and Methoxyl Group in Bamboo MWL before and after Methanolysis of the MWL

Methanolysis	OCH_3		p-Coumaric acid, %[b]
	%[a]	Per C_9	
Before	14.5	0.94	5.5
After[c]	21.5	1.40	5.1
After[d]	25.3	1.64	5.3

[a] Methoxyl content is expressed as percent on MWL.
[b] p-Coumaric acid content is expressed as percent on the original or the methylated MWL preparations.
[c] The methanolysis was carried out at room temperature for 3 days.
[d] The methanolysis was carried out by refluxing MWL in methanolic hydrogen chloride (1%) containing anhydrous dioxane for 3 hr.

Table III. Comparison of p-Coumaric Acid Content in Various Lignin Preparations before and after Thioglycolic Acid Treatment

Lignin	Before treatment		After treatment		
	$(OCH_3)/C_9$[a]	p-CA, %[b]	S, %[c]	SCH_2COOH/C_9[d]	p-CA, %[e]
Bamboo MWL	0.94	5.5	10.00	0.87	6.1
Bamboo BNL[f]	0.98	8.5	9.60	0.80	7.6
Juzuldama MWL	0.90	10.4	8.74	0.74	9.2

[a] Methoxyl content was determined by the conventional method and expressed as number of moles per C_9 unit (*14*).
[b] Content of p-coumaric acid.
[c] Content of sulfur in thioglycolic acid lignin.
[d] Number of moles of thioglycolic acid group combined with lignin which was calculated from analytical data of elementary composition.
[e] Content of p-coumaric acid is expressed as percentage based on thioglycolic acid lignin.
[f] Brauns' native lignin was prepared from sawdust of bamboo by the conventional method (*24*).

before and after thioglycolation. Thioglycolic acid reacts more readily with α-carbon atoms than does methanol, because even the phenylcoumarane ring which is stable on methanolysis is cleaved by thioglycolic acid (22). The α-position was attacked by thioglycolic acid at a ratio of 0.74 to 0.87 mole per C-9 unit, as calculated from the sulfur content. Nevertheless, the contents of PCA retained in the thioglycolated MWL preparations are approximately equal to those in the untreated

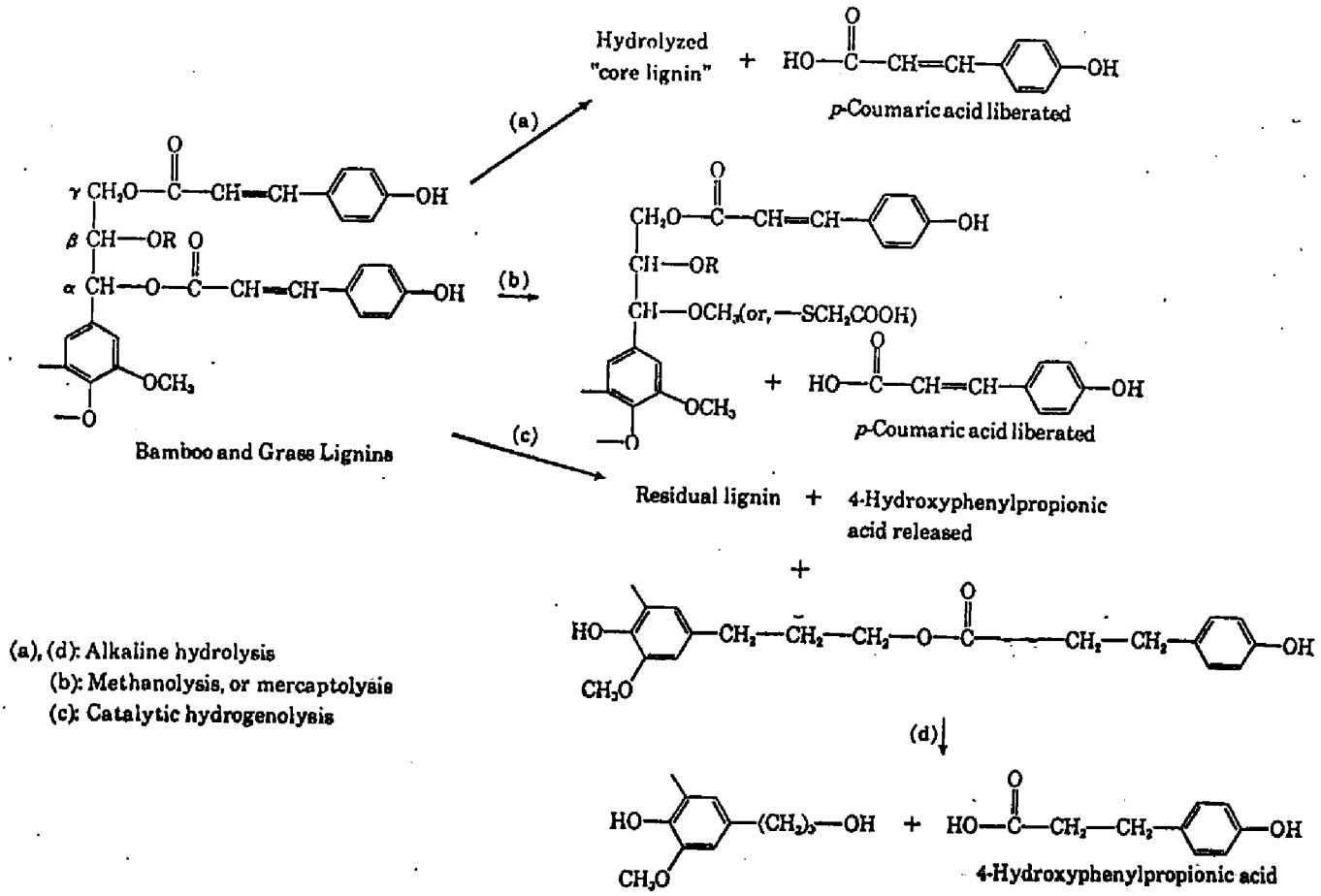
preparations. These results are quite in accordance with those obtained from methanolysis experiment, indicating that PCA is not esterified at the α-position.

Qualitative experiments with catalytic hydrogenolysis:

Benzyl ether and ester groups can be reduced by hydrogenolysis with Pd-charcoal, yielding the corresponding alcohol and acid, respectively (16). Therefore, GGT was employed for this catalytic hydrogenolysis as a model substance. It turned out to be impossible, however, to split off selectively the acetyl group at the α-postion of GGT by this procedure, because all proton signals due to the three acetyl groups were still observed without any changes in the NMR spectrum after hydrogenolysis. Also, MWL was found to change only slightly on hydrogenolysis carried out under the more drastic conditions described under "Materials and Methods". Such difficulties in the degradation of lignin by this type of hydrogenolysis were also reported by Adler (23). The residual lignin recovered showed the same UV-spectrum as that of untreated lignin. However, the fact that ether-soluble hydrogenolysis products were obtained, although in small amounts (about 10%), indicates that the products originated as a result of cleavage of ether structures at the α- or β-positions of the lignin polymer. Accordingly, the ethers attached to the α-position could also be split off on hydrogenolysis. From the acid fraction of the products, 4-hydroxyphenylpropionic acid, probably derived exclusively from PCA esters, was isolated by TLC (Rf 0.50). However, it is not clear whether it was liberated by hydrogenolytic cleavage alone or by hydrolytic cleavage of the esters. This compound was also obtained

after alkaline hydrolysis of the phenolic fraction and identified with 4-hydroxyphenylpropionic acid by UV-spectrum. At the same time, a strong absorption band due to ester carbonyl was observed in the IR-spectrum of the phenolic fraction. Therefore, 4-hydroxyphenylpropionic acid contained in the phenolic fraction is linked in the ester form to the terminal γ-carbon but not to the α-carbon atoms.

Outlines of the chemical analyses employed for this investigation are given in Scheme I.



Scheme I. Chemical analyses of bamboo and grass lignins.

In conclusion, analytical data described so far support the possibility that the majority of PCA molecules in bamboo and grass lignins are linked to the γ-position of the side chain of lignin molecules. However, more appropriate model experiments must be carried out, and the investigation is in progress to obtain definite evidence for the structural pattern of the ester linkages of PCA in lignins.

As for the biochemical formation of PCA esters in graminae plant lignins, two possibilities are considered:

1. p-Coumaroyl group may be enzymatically transferred to the terminal γ-carbons of oligomers of DHP or growing lignin polymers.

2. Coniferyl-γ-p-coumarate may participate in lignification.

However, the former is considered more likely than the latter on the grounds that coniferyl-γ-p-coumarate has not yet been found in graminae plants.

MATERIALS AND METHODS

Preparation of MWL:

Stems of mature graminae plants such as Juzudama (Coix lachryma), Susuki (Miscanthus condensatus), Ogi (Miscanthus florus), Ashi (Phrogmites communis), Danchiku (Arundo donax), and bamboo (Phyllostachys pubescens) were used for the preparation of MWL.

Ten grams of extractive-free plant powder was ground for 48 hr in dry toluene with a vibratory ball mill (Siebtechnik GH, Mühlheim,Germany) except that Juzudama was ground in the absence of toluene (11). MWL was extracted with 90% aqueous

dioxane and purified according to the standard method of Björkman (12).

Preparation of dehydrogenation polymer:

Coniferyl alcohol (600 μmoles) was dissolved in 100 ml of distilled water containing 1 ml of EtOH, and horse radish peroxidase (0.5 mg) was added to the solution. Then, 20 ml of 0.5% hydrogen peroxide solution was added to the reaction mixture in 0.5-ml portions at 10-min intervals with continuous stirring. After 24 hr small amounts of peroxidase and 0.5% hydrogen peroxide were added to the reaction mixture, which was stirred for an additional 24 hr. Precipitated DHP was collected by centrifugation and washed with water. After complete drying, the precipitate was dissolved in a minimum amount of dioxane and precipitated into ether. The precipitated DHP was again collected by centrifugation and dried over P_2O_5 (yield 60-70%).

In order to examine effect of organic acids upon ester formation, acetic, propionic, n-butyric, acrylic, succinic, and citric acids were individually added to the reaction mixtures in equimolar concentrations to that of coniferyl alcohol, and cinnamic, p-coumaric, and ferulic acids-2-^{14}C in half-molar concentration. These reaction mixtures were adjusted to pH 5.5 with $NaHCO_3$. The various DHP preparations thus obtained were used for measurement of IR- and UV-spectra, determination of the acids incorporated into DHP, and other analytical experiments. IR-spectra were taken in disks of KBr with Nippon Bunko DS-2 IR spectrophotometer.

Measurement of pH-UV Absorbancy curves for model compounds and various MWL preparations:

PCA, ethyl p-coumarate, and p-methoxycinnamic acid were used as model compounds for measurement of their pH-UV absorbancy curves. These compounds give the absorbance minima and maxima in acid and alkaline solutions shown in Table IV. On the basis of these data the absorbances of PCA, ethyl p-coumarate, and p-methoxycinnamic acid at varying pH values were taken at 335,353, and 309 nm, respectively. In this case the concentrations of model compounds were kept equal to compare changes in absorbance with variation of pH. In the same manner, the absorbancy curves for MWL preparations were taken at 355 nm, corresponding to their maximal absorbance. In this case, however, dioxane was added in a concentration of 50% to all buffer solutions in order to dissolve the MWL.

Table IV. UV Spectral Data of Model Compounds

	0.1N HCl		*0.1N NaOH*	
Model compounds	λ_{min}	λ_{max}	λ_{min}	λ_{max}
p-Coumaric acid	250	310	260	335
Ethyl *p*-coumarate	250	310	268	353
p-Methoxycinnamic acid	240	282	248	309

Methanolysis of GGT, radioactive DHP, and bamboo MWL:

GGT (100 mg) was dissolved in 5 ml of 0.5% methanolic hydrogen chloride and the solution was allowed to stand at room temperature for 2 days according to the method of Adler (13). Then, the reaction mixture was neutralized with $NaHCO_3$, evaporated to dryness in vacuo, and the methoxylated preparation transferred into $CHCl_3$, which was washed with water and dried over Na_2SO_4. After evaporation of the chloroform, the methoxyl content of the residue was determined according to the conventional method (14).

Bamboo MWL (200 mg) was dissolved in 10 ml of mixture of anhydrous dioxane-1% methanolic hydrogen chloride (1:1), and the reaction mixture was allowed to stand at room temperature for 4

days (13). The MWL was recovered and the content of methoxyl groups of the recovered MWL determined, paralleling the determination of PCA esters retained in the MWL. Similarly, radioactive DHP (10 mg) was submitted to the methanolysis. The methanolysis products were examined by radiochromatography as described below.

Thioglycolation of various lignin preparations:

Each lignin preparation (200 mg) was dissolved in 2 ml of anhydrous thioglycolic acid containing 0.2 ml of boron trifluoride-diethylether complex (15) and the reaction mixture was allowed to stand at room temperature for 4 days. Then, the reaction mixture was dropped into ether with stirring, and the precipitated thioglycolated lignin was obtained by centrifugation, dissolved in a minimum amount of dioxane, and again precipitated into ether. Thioglycolated lignins thus obtained were employed for elemental analysis, measurement of their UV-spectra and determination of PCA esters retained.

Determination of PCA esters contained in MWL:

Each lignin preparation (10 mg), such as untreated bamboo and Juzudama MWL, the corresponding methanolyzed or thioglycolated lignins, and the DHP containing radioactive cinnamic acid, was hydrolyzed in N NaOH solution at room temperature for 2 days. After acidification of the hydrolyzed mixture, the organic acids were extracted with ether. The ether was quantitatively subjected to paper chromatography, using a solvent system of toluene-AcOH-H_2OH (4:1:5, upper layer). PCA observed on the chromatograms under UV light in ammonium vapor was eluted with 5 ml of 95% EtOH at 50° for 15 min. The content of the

acid was determined by measurement of its absorbance at 310 nm. Contents of cinnamic acids-2-^{14}C incorporated into DHP were calculated from radioactivities of DHP, which were measured with a gas flow counter or with a Beckman liquid scintillation counter (LS-100) in toluene containing PPO (40 mg/10 ml) and POPOP (0.2 mg/10 ml).

Radiochromatography of cinnamic acid-2-^{14}C and PCA-2-^{14}C:

Radioactive DHP (10 mg) was subjected to alkaline hydrolysis or methanolysis as described above. Ether-soluble acid fractions were examined by PPC with the solvent system, n-butanol-EtOH (5:1) saturated with 1.5 N NH_4OH for detection of labeled cinnamic acid, and with the solvent system, $CHCl_3$-AcOH-H_2O (2:1:1, lower layer) for detection of labeled PCA. Radioactivities on the chromatograms were scanned with a radiochromatogram scanner (Aloka DCS-4) or a Beckman scintillation counter by the paper strip method.

Catalytic hydrogenolysis:

GGT (100 mg) dissolved in 40 ml of EtOH was subjected to catalytic hydrogenolysis in the presence of H_2 and 100 mg of Pd-charcoal (5%) at room temperature for 1 hr (16). MWL (100 mg) dissolved in 20 ml of dioxane was treated in the same way. Alternatively, a more drastic catalytic hydrogenolysis was carried out with Juzudama MWL (17): MWL (500 mg) at 80 atm and at 80-100° for 5 hr. After recovering the residual lignin by precipitation into ether, the hydrogenolysis products were fractionated into ether-soluble acid- and phenolic fractions. The acid fraction was examined by TLC with a solvent system of toluene-ethylformate-HCOOH (5:4:1). The phenolic fraction was

used for measurement of IR-spectrum to examine the presence of ester carbonyl groups. After alkaline hydrolysis of this fraction, the hydrolyzates were examined by TLC in order to detect liberated 4-hydroxyphenylpropionic acid.

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CHAPTER VII

CONCLUDING REMARKS

G-6-P- and 6-PG dehydrogenases involved in pentose phosphate pathway, dehydroquinate hydro-lyase and dehydroshikimate reductase in shikimate pathway, and O-methyltransferase in cinnamate pathway were isolated from the shoots of growing bamboo. The properties of these enzymes were investigated in relation to lignification of the bamboo shoots.

It was found that, during the lignification, the enzymes such as O-methyltransferase and phenylalanine ammonia-lyase were activated rather than the enzymes in pentose phosphate and shikimate pathways that are situated at the early stages on the metabolic pathways to lignin.

The enzyme works performed so far supported the biosynthetic pathways obtained with tracer techniques (CHAPTER II and III).

Ferulic acid-O$^{14}CH_3$ was first used for the study on biosynthesis of syringyl lignin in order to find a clue to the problem whether or not ferulic acid actually incorporated into syringyl units of lignin with retention of the methyl group. From the analytical data obtained by nitrobenzene oxidation and ethanolysis of the plants fed with various labeled compounds, it was found that ferulic acid-O$^{14}CH_3$ was incorporated into syringyl units as well as into guaiacyl units. This finding supports the early indication that ferulic acid may serve as a syringyl lignin precursor (CHAPTER IV).

It is well known that angiosperm lignins consist of both guaiacyl and syringyl units whereas gymnosperm lignins hardly contain syringyl units but guaiacyl units. However, it is unknown what biochemical factors are involved in the occurrence of such differences in methoxyl patterns. In order to elucidate this problem O-methyltransferase was for the first time cell-free extracted from gymnospermous plants such as pine seedlings and ginkgo shoots. On the basis of the comparative studies on the substrate specificities of O-methyltransferases from bamboo, poplar, pine, and callus tissues of Salix caprea and mulberry (Morus bombycis), it was concluded that O-methyltransferase is one of the key enzymes that are involved in formation of syringyl lignin. Because O-methyltransferases extracted from bamboo, poplar and the callus tissues utilized both caffeic and 5-hydroxyferulic acids as substrates, yielding ferulic (guaiacyl unit) and sinapic (syringyl unit) acids, respectively, whereas the enzymes from pine and ginkgo were found to methylate caffeic acid alone as a rule (CHAPTER V).

The esters of p-coumaric acid contained in bamboo and grass lignins are considered as a biological feature because such esters are not found in other species of higher plants. However, their chemical structure is not yet established .

In order to determine the position (α or γ) of p-coumarate esters associated with the side chain of lignin molecules, it was found to be useful to utilize methanolysis, thioglycolysis and hydrogenolysis as analytical methods. The analytical data obtained with model substances and natural lignins led the author to a conclusion that the majority of p-coumaric acid molecules are linked to the terminal γ- position of side chain of lignin. (CHAPTER VI)

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